DEPARTMENT OF MEDICINE, CARDIOLOGY UNIT
KAROLINSKA UNIVERSITY HOSPITAL
Karolinska Institutet, Stockholm, Sweden

# STUDIES ON THE REGULATION OF ENDOTHELIAL NITRIC OXIDE SYNTHASE IN ENDOTHELIAL DYSFUNCTION

Ruha Cerrato


Karolinska Institutet

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"Split the atom's heart, and lo!
Within it thou wilt find a sun…"

*Persian mystic poem*

To Mahan, Jayden and Corinne

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# ABSTRACT

Ischaemic heart disease and cerebrovascular disease are the leading causes of morbidity and mortality in the world. The underlying progression of the disease is linked to a reduction in the bioavailability of nitric oxide. One factor contributing to this is an increase in the production of superoxide radicals. A combination of increased oxidative stress, inappropriate lipid metabolism and cell death sets the stage for what will subsequently develop into atherosclerosis. The process of atherogenesis can slow down if patients at risk are identified early, receive the necessary pharmacological treatment and change to a healthier lifestyle. The aim of the following studies was to identify whether the uptake, synthesis and recycling of tetrahydrobiopterin (BH4), the essential co-factor of endothelial nitric oxide synthase (eNOS), could influence oxidative stress in human vasculature. We also sought to elucidate whether endothelin-1 (ET-1), a potent vasoconstrictor, played an important role in oxidative stress in human vasculature and the potential mechanisms underlying this influence.

In Study I, 49 patients with coronary artery disease took part in a placebo-controlled clinical trial with the aim of determining the mechanisms of exogenous BH4 in relation to vascular function. Oral BH4 treatment significantly elevates the levels of BH4 in blood, but this effect is limited by the rapid systemic oxidation of exogenous BH4. The ratio of reduced to oxidised biopterins in blood and vascular tissue is unchanged by exogenous BH4 treatment, resulting in no net effect on vascular superoxide production or endothelial function.

In Study II, the aim was to explore the regulation of endogenous BH4 and subsequent effects on endothelial function in patients with coronary artery disease. In three clinical models and one *in vitro* model, involving 465 subjects, we observed that an inability to increase vascular BH4 synthesis leads to significant impairment of endothelial function.

In Study III, the aim was to explore the role of ET-1 in endothelial dysfunction, specifically with regard to superoxide production. ET-1 increases superoxide production in human coronary artery bypass grafts via a receptor-driven mechanism involving, the largest contributor of superoxide in the vascular wall, nicotine amide dinucleotide phosphate (NADPH) oxidase.

In Study IV, I applied what I had learned from Study I and Study II and sought to further delineate whether endothelin-1 influences biopterin homeostasis in both human and animal tissues. ET-1 did not have any effect on BH4 in human coronary artery bypass grafts or resistance arteries, endothelial cells and mice with an over-expression of ET-1 in the endothelium (ET-transgenic mice).

# SAMMANFATTNING

Ischemisk hjärtsjukdom utgör den vanligaste orsaken till sjukhusvård och död i världen. Otillräcklig blodförsörjning av hjärtat till följd av blodpropp eller åderförkalkning (ateroskleros) i hjärtats kranskärl är huvudorsaken till mortalitet och morbiditet. Ett förstadium till ateroskleros är endoteldysfunktion. En av de viktigaste molekylerna som endotelet frigör är kväveoxid (NO). NO bevarar kärlets förmåga att vidga sig adekvat, förhindrar blodproppsbildning och inflammation. En annan viktig substans är endotelin-1 (ET-1), en peptid som i huvudsak agerar i motsatsförhållande till NO. NO skyddar vävnaden och ökar blodflödet medan endotelin minskar blodflödet och agerar vävnadsskadande. En huvudsaklig orsak till minskad biotillgänglighet av NO är ökad produktion av fria radikaler såsom superoxid.

I denna avhandling har jag huvudsakligen studerat det enzym som producerar NO, endothelialt kväveoxidsyntas (eNOS) och närmare bestämt den ko-faktor (tetrahydrobiopterin eller BH4) som huvudsakligen avgör huruvida eNOS ska producera NO eller superoxid, då detta enzym kan vara en källa för båda.

I studie I randomiserades 49 patienter med koronarsjukdom som stod på väntelista för bypass kirurgi till att få BH4 oralt eller placebo. Patienterna erhöll 2-6 v behandling med BH4 eller placebo. Före och efter behandlingen testades kärlfunktionen. Oralt BH4 förbättrade inte kärlfunktionen. Kärlbitar från operationen studerades och en ökning av BH4 och dess biprodukt BH2 i vener observerades. Upptaget av BH4 ökade inte i artärer. Detta resulterade i utebliven förändring i förhållandet mellan BH4 och dess biprodukter och därför heller ingen minskning på superoxidproduktion, dvs eNOS fortsatte att producera superoxid. Sammanfattningsvis visade studien att tillförsel av oralt BH4 ökar tillgängligheten av BH4 men även BH2, vilket totalt sett resulterar i utebliven förbättring av kärlfunktionen.

Studie II består av fyra delar, i första delen deltog friska försökspersoner, de randomiserades till vaccination med salmonella typhi eller placebo och kärlfunktionen mättes före och efter vaccinationen. Vaccinationen resulterade i en ökning av BH4 , interleukin 6 och c-reaktivt protein i plasma. Kärlfunktionen försämrades akut av vaccinationen. I del två studerades 440 patienter med kranskärlsjukdom avseende förekomst av en speciell haplotyp av guanosine cyclohydrolas (GCH) genen och vi fann att denna var kopplad till endoteldysfunktion samt förhöjt c-reaktivt protein. I del tre delades patienter med kranskärlssjukdom upp i en grupp med homozygoter för denna GCH haplotyp (XX) och en grupp utan denna haplotyp (OO). Dessa fick sedan vaccination med salmonella typhi och kärlfunktion mättes. Patienter med haplotypen XX kunde inte öka plasma BH4 och hade en försämrad kärlfunktion efter vaccination. I del fyra användes en kärlmodell som jag utvecklade för att studera hur kärlet kan producera eget BH4 och hur denna process påverkas av inflammatoriska substanser. I en sådan miljö ökar BH4 i kärlet och detta är direkt kopplat till förbättrad endotelfunktion.

I studie III studerade jag endotelin-1 och huruvida denna peptid kan öka bildningen av fria radikaler i kärl från patienter med kranskärlssjukdom. Endotelin-1 ökar produktionen av superoxid och jag kunde härleda detta till att vara medierat via endotelinreceptorer och NADPH oxidas som huvudsaklig superoxidkälla.

I studie IV studerade jag vidare direkta effekter av ET-1 på BH4, den essentiella ko-faktorn till eNOS. I denna studie visar jag i olika cell, djur och kärlmodeller att endotelin-1 inte har någon effekt på tillgängligheten av BH4 i vävnaden och således är eNOS inte en huvudsaklig källa för superoxidbildning i detta sammanhang.

# LIST OF PUBLICATIONS

I. C Cunnington, T. Van Assche, C Shirodaria, I Kylintireas, AC Lindsay, JM Lee, C Antoniades, M Margaritis, R Lee, **R Cerrato**, MJ Crabtree, JM Francis, R Sayeed, C Ratnatunga, R Pillai, RP Choudhury, S Neubauer, KM Channon. Systemic and vascular oxidation limits the efficacy of oral tetrahydrobiopterin treatment in patients with coronary artery disease. Circulation. 2012 Mar 20;125 (11):1356-66.

II. C Antoniades, C Cunnington, A Antonopoulus, M Neville, M Margaritis, M Demesthenus, J Bendall, A Hale, **R Cerrato**, D Tousolis, C Bakogiannis, K Marinou, M Toutouza, C Vlachopoulos, P Leeson, C Stefanidis, F Karpe, KM Channon. Induction of vascular GTP-cyclohydrolase 1 and endogenous tetrahydrobiopterin synthesis protect against inflammation-induced endothelial dysfunction in human atherosclerosis. Circulation 2011 Oct 25;124(17):1860-70.

III. **R. Cerrato**, C. Cunnington, M.J Crabtree, C. Antoniades, J. Pernow, K.M Channon, F.Böhm. Endothelin-1 increases superoxide production in human coronary artery bypass grafts. Life Sci. 2012;91:723-8.

IV. **R.Cerrato,** M.J Crabtree, A. Hale, C. Antoniades, N. Alp, K. Kublickiene, E.Schiffrin, K.M Channon, F.Böhm. Role of eNOS uncoupling in endothelin-1 mediated superoxide production. Manuscript submitted.

# LIST OF ABBREVIATIONS

| | |
|---|---|
| 5-HT | 5-hydroxytryptamine or serotonin |
| ACH | Acetylcholine |
| B | Biopterin |
| BH2 | Dihydrobiopterin |
| BH4 | 5,6,7,8 tetrahydrobiopterin |
| CABG | Coronary artery bypass grafting |
| CK2 | Casein kinase 2 |
| DAHP | Diamino-6-hydroxypyrimidine |
| DHE | Dihydroethidium |
| DHFR | Dihydrofolate reductase |
| DHPR | Dihydropteridine reductase |
| DPI | Diphenyleneiodonium |
| DTE | Dithioerythrotiol |
| EDTA | Ethylene diamine tetra-acetic acid |
| eNOS | Endothelial nitric oxide synthase |
| ET-1 | Endothelin-1 |
| FMD | Flow-mediated dilation |
| GPCR | G-protein coupled receptor |
| GTPCH | Guanosine triphosphate (GTP) cyclohydrolase-1 |
| HEPES | 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid |
| HPLC | High-pressure liquid chromatography |
| HUVEC | Human umbilical vein endothelial cell |
| IL-6 | Interleukin-6 |
| IMA | Internal mammary artery |
| L-NAME | $N^G$-nitro-L-arginine methyl ester hydrochloride |
| MAPK | Mitogen activated protein kinase |
| MMP | Matrix metalloproteinases |
| MMP-9 | Matrix metalloproteinase 9 |
| MRI | Magnetic resonance imaging |
| MTX | Methotrexate |
| NADPH | Nicotine amide dinucleotide phosphate |
| NE | Norepinephrine |
| NF-κB | Nuclear factor κB |
| NO | Nitric oxide |
| NOX | NADPH oxidase |
| PAH | Pulmonary artery hypertension |
| PARP | Poly (ADP ribose) polymerase |
| PASMC | Pulmonary aortic smooth muscle cell |
| ROS | Reactive oxygen species |
| SEND | Murine endothelial celline |
| SNP | Sodium nitroprusside |
| SR | Sepiapterin reductase |
| SV | Saphenous vein |
| TNFα | Tumor necrosis factor α |
| VSMC | Vascular smooth muscle cells |

# 1 INTRODUCTION

## 1.1 ENDOTHELIAL DYSFUNCTION AND CARDIOVASCULAR DISEASE

Ischaemic heart disease and cerebrovascular disease are the leading causes of morbidity and mortality in the world. An inadequate or obstructed blood supply to the heart is the cause of ischaemia. Endothelial dysfunction refers to the initial stages of the process leading to the development of atherosclerosis and is mainly due to the reduced bioavailability of nitric oxide (NO) and increased levels of the vasoconstrictor, endothelin-1 (ET-1).[1]

Generally, four major mechanisms are described as reducing the bioavailability of NO:

(1) Reduced endothelial nitric oxide synthase (eNOS) expression
(2) A lack of substrate (L-arginine) or co-factors (mainly the essential co-factor, tetrahydrobiopterin; BH4) for eNOS or the presence of antagonist (asymmetric dimethylarginine)
(3) Reduced eNOS activation and
(4) Increased consumption of NO due mainly to the increased production of superoxide radicals

Risk factors for cardiovascular disease such as smoking, aging, hypercholesterolemia, hypertension, hyperglycaemia and a family history of early atherosclerotic disease are all associated with the reduced bioavailability of NO and the subsequent loss of endothelium-dependent vasodilatation.[2] Several studies have now proven that endothelial dysfunction is a strong and independent predictor of cardiovascular events.[3]

The identification of subjects at risk before clinically evident atherosclerosis is essential for the correct management in primary prevention. In this situation, the measurement and assessment of endothelial function may be valuable in identifying subjects at risk, as well as evaluating treatment effects both in research and in clinical practice.

In the following chapters, I shall review the sources and mechanisms of superoxide production in human vasculature and discuss the importance of these sources in relation to clinical practice. I will also highlight the role of ET-1 as a cause of endothelial dysfunction based on superoxide production via its interaction with different sources of superoxide.

## 1.2 BIOLOGICAL ACTIONS OF REACTIVE OXYGEN SPECIES

The common denominator of all reactive oxygen species (ROS) is that they originate from oxygen. As we are immersed in an oxygen-rich environment, it is not surprising that we are bound to experience the formation of oxygen radicals. They form as a by-product of the electron transport of aerobic respiration in mitochondria or by oxidoreductase enzymes. These radicals perform important biological functions such as host defence, the biosynthesis of hormones and fertilisation and act as intracellular signaling molecules.
ROS are usually divided into two groups:

(1) free radicals, such as superoxide, hydroxyl and NO, and
(2) non-radical derivatives of oxygen, such as hydrogen peroxide and peroxynitrite.[4]

Oxygen is first reduced to superoxide and superoxide dismutase then promotes further reduction to hydrogen peroxide, which can react with metals and form hydroxyl radicals. Peroxynitrite is formed through the reaction of superoxide with NO, leading to the reduced bioavailability of NO, which in turn leads to endothelial dysfunction. One pathway illustrating the role of peroxynitrite formation is through the nitration of prostacyclin synthase and by the inhibition of soluble guanylyl cyclase. This is the bedrock upon which the link between oxidative stress and endothelial dysfunction rests.

When superoxide production exceeds innate antioxidant defence systems, cellular damage starts to occur such as for example the inhibition of cell growth, the promotion of apoptosis or cell death.[5] Lipid peroxidation, protein oxidation and DNA damage are usually attributed to ROS and they are involved in atherogenesis. Oxidative modifications of proteins may result in the formation of nitrotyrosine, which represents a marker of cardiovascular disease.[6 7] In the atherosclerotic process, ROS can promote oxidised low-density lipoprotein formation, stimulate matrix metalloproteinases, increase vascular smooth muscle cell growth and provoke inflammatory mediator production, including matrix metalloproteinase-1, intercellular adhesion molecule 1 and vascular cellular adhesion molecule 1. The reduced production of superoxide leads to a decrease in the recruitment of macrophages to the endothelial surface, which will have direct effects on the formation of the atherosclerotic plaque.[8]

### 1.2.1 Superoxide signaling in cardiovascular diseases and clinical implications

The links between superoxide signaling and cardiovascular disease states such as coronary artery disease and myocardial infarction have been described in a large set of animal studies. [9] Through the genetic deletion or overexpression of major superoxide sources, such as the NADPH oxidase system or antioxidant defence systems such as superoxide dismutases, a large body of knowledge has been retrieved in animals and cell models.[10] For the past decade or so, some of these mechanisms have been studied in humans. Some might argue that oxidative stress is not linked to the progression of atherogenesis in human, as almost all large clinical trials with oral antioxidants have failed to have any influence on hard endpoints and clinical outcomes.[11] The results of these trials indicate that oxidative stress is not a one-directional process. The cascade of reactions initiated by superoxide interacting with pro-oxidative enzyme systems and an equally wide range of antioxidant defence mechanisms makes the arena complex. In this thesis we sought to find mechanistic explanations to the link between superoxide production and endothelial dysfunction.

## 1.3 SOURCES OF HUMAN VASCULAR REACTIVE OXYGEN SPECIES

The major science underlying our current knowledge of ROS generation in human vasculature stems from studies of cultured endothelial, smooth muscle cells and coronary artery bypass grafts. The use of bypass grafts has the advantage of being

(1) accessible for *ex vivo* mechanistic and functional studies and
(2) obtained from a patient with manifest atherosclerosis.

In 2004, Guzik *et al*. verified NADPH oxidase as the main contributor to ROS generation in human saphenous veins. An increase in the expression of the subunits of NADPH oxidase was found in vessels from diabetic patients.[12] There was also evidence of contribution by

eNOS to the overall ROS burden, especially in diabetics. It has been proposed that NADPH oxidase and eNOS are involved in intricate crosstalk. In many review articles, initial ROS generation from NADPH oxidase is described as a trigger resulting in a series of reactions, including the activation of the other enzyme systems.[13] These other sources of superoxide are mitochondrial ROS and xanthine oxidase (Fig 1).

### 1.3.1 Endothelial nitric oxide synthase

The role of eNOS in the development of endothelial dysfunction attracted renewed interest when it was understood that this enzyme generates superoxide. The rationale behind this phenomenon is now well known. The family of NOS enzymes, eNOS, inducible NOS and neuronal NOS, all share an essential need for co-factors BH4, NADPH and the flavins and flavin mononucleotide, on top of the substrate L-arginine, to generate NO. Nitric oxide synthases are homo dimers and each monomer consists of a reductase domain with binding sites for NADPH, flavins, flavin mononucleotide and calmodulin and an oxygenase domain containing an iron heme group and binding sites for L-arginine and BH4. Starting from NADPH, electrons flow to the flavins in the reductase domain, to the iron of the heme in the oxygenase domain. Calmodulin regulates electron flow between the reductase and oxidase domain. BH4 appears to be essential to donate electrons to the heme group in the oxygenase domain in order to oxidise L-arginine. In the absence of BH4, electron flow from the reductase domain is diverted towards molecular oxygen rather than L-arginine, resulting in superoxide production rather than NO synthesis. This is the main explanation of eNOS uncoupling. In the following sections, I will further discuss the role of BH4 as a key regulator of eNOS.

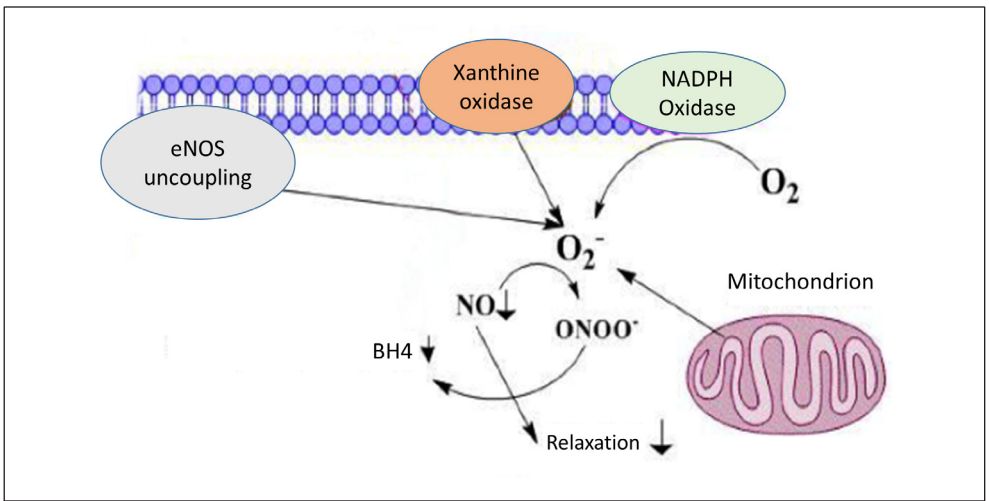


Fig 1. The largest contribution of vascular superoxide ($O_2^-$) originates from nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, which is membrane bound. Reactive oxygen species (ROS) generated from NADPH oxidase reduce the bioavailability of nitric oxide (NO) due to the reaction of NO with $O_2^-$ generating peroxynitrite. Peroxynitrite oxidizes tetrahydrobiopterin (BH4), leading to reduced BH4 and eNOS uncoupling, which means that eNOS produces superoxide instead of NO. Xanthine oxidase and mitochondria also contribute to ROS generation in the cell.

### 1.3.2 BH4 and its role as a key regulator of NOS

BH4 belongs to the family of chemical structures called pteridines and it was first described in 1895.[14] The first description of BH4 was in relation to its role as a co-factor of four aromatic amino acid hydroxylases (tyrosine hydroxylase, phenylalanine hydroxylase, tyrosine hydroxylase and tryptophan hydroxylase). BH4 is therefore involved in the synthesis of epinephrine, norepinephrine, dopamine and 5-hydroxytryptamine (5-HT or serotonin). It took several decades (1989) for Stuehr *et al.*[15] to describe BH4 as a co-factor for NOS enzymes.

#### *1.3.2.1 Regulation of NOS*

A turning point in NO research was the finding that NOS enzymes contain a cytochrome P450 type heme which enables the activation of oxygen without the co-factor.[14 16] BH4 functions as an allosteric modulator of arginine binding; i.e. when BH4 is present, the affinity for binding arginine to NOS increases. In a pro-inflammatory environment with increased oxidative stress, BH4 can be oxidised by peroxynitrite to the non-protonated trihydrobiopterin (BH3) radical and then to dihydrobiopterin (BH2) and biopterin (B).

In the literature, the question of whether BH4 can be oxidised by other ROS has also been raised. Due to the chemical properties of these reactants, if superoxide, NO and BH4 are present, superoxide will react with NO, first forming peroxynitrite and then the further oxidation of BH4. [17]

BH2 has been shown to compete with BH4 to bind to NOS. In endothelial cells with oxidative stress, the electron transfer become uncoupled from L-arginine oxidation, leading to superoxide being produced from the oxygenase domain (Fig 2). In this setting, BH4 and particularly the ratio of BH4:BH2+B become key determinants of eNOS uncoupling and, in the long run, eNOS regulation.[16]

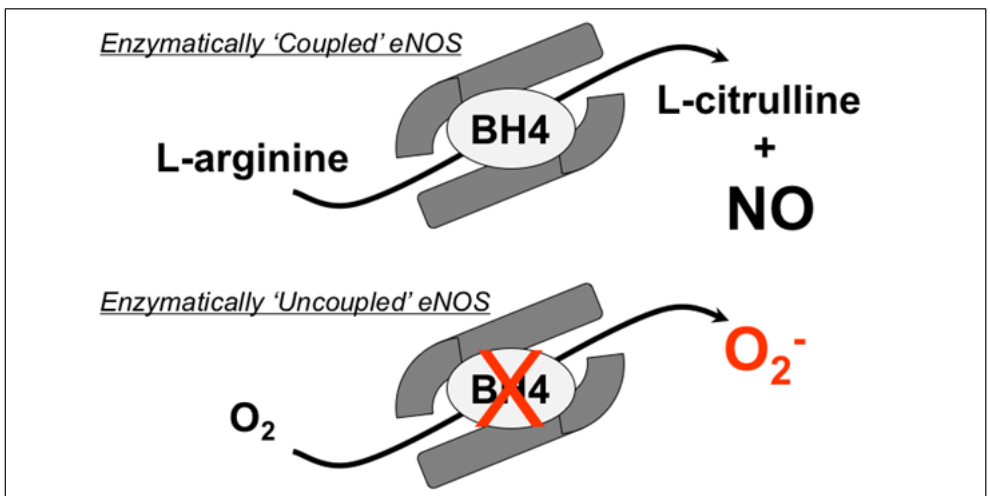


Fig 2. In conditions of low superoxide and an abundance of L-arginine, endothelial nitric oxide synthase (eNOS) produces NO and L-citrulline. In conditions of increased superoxide production and the formation of peroxynitrite, BH4 is oxidised, resulting in the reduced bioavailability of BH4, the uncoupling of eNOS and production of superoxide.

Another aspect of eNOS regulation, which will not be dealt with any further in this thesis, is whether it is possible that eNOS uncoupling can occur due to a lack of substrate such as L-arginine. In cell-free systems, using electron spin trapping, it was reported that L-arginine alone is unable to inhibit superoxide release from BH4 free eNOS,[18] whereas the addition of BH4 was able to reduce superoxide formation without L-arginine.[19] In endothelial cells, the addition of L-arginine in the absence of BH4 increased eNOS-mediated ROS.[20] Furthermore, animal studies in which eNOS coupling is restored with the emphasis on L-arginine availability have failed to take into account the importance of quantified BH4 and BH2.[21]

*1.3.2.2 De novo synthesis of BH4 and relation to inflammatory stimuli*

The *de novo* synthesis of BH4 (Fig 3) involves the actions of the rate-limiting enzyme, guanosine triphosphate cyclohydrolase 1 (GTPCH), encoded by the GCH-1 gene. Animal and elegantly designed in-vitro models show a direct correlation between GCH-1, GTPCH protein and levels of intracellular BH4.[22] One such animal model is the hph-1 mouse with constitutively reduced expression of GCH-1. The hph-1 mouse model was produced in 1988 by screening N-ethyl-N-nitrosurea-treated mice for the presence of hyperphenylalaninemia.[23] In the hph-1 mouse, the relative quantification of GCH-1 mRNA expression correlate directly with the protein level and enzymatic activity of GTPCH, along with the reduced synthesis of BH4.[22] In humans, the relationship becomes more complex and even more so in a pro-inflammatory environment.

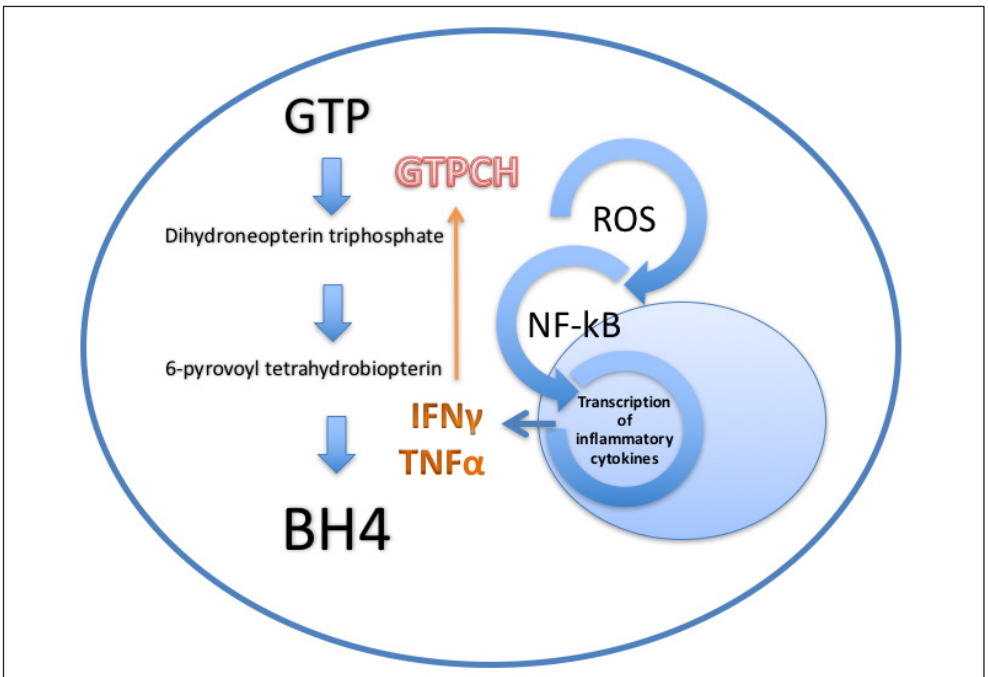


Fig 3. The *de novo* synthesis of BH4 is driven by the rate-limiting enzyme, GTP cyclohydrolase 1 (GTPCH). In a pro-oxidative setting, GTPCH activity and expression is increased due to the influence of inflammatory cytokines such as interferon γ (IFN γ) and tumor necrosis factor α (TNFα). Reactive oxygen species (ROS) activate nuclear factor kappa B (NF-κB) signalling.

The regulation of the GCH-1 gene with regards to the influence of inflammatory cytokines has been studied in endothelial cells[24], leukocytes[25] and smooth muscle cells.[26] In these studies tumor necrosis factor α (TNFα), interleukin-6, lipopolysaccharide (LPS) and hydrogen peroxide stimulate BH4 synthesis through increased GCH-1 mRNA. The underlying mechanisms have been attributed to the increased activation of NF-κB and the janus kinase signal transducer activator of transcription protein (JAK-STAT), which are key inflammatory transcription factors.[14]

Another pathway which is linked to the process of atherogenesis in the vasculature involves the influence of shear stress on GTPCH regulation. Widder *et al.*[27] were able to show that GTPCH activity in human endothelial cells was increased 30 fold in areas of laminar shear stress, leading to a proportionately significant increase in BH4. The mechanism was the casein kinase 2 (CK2)-dependent phosphorylation of GTPCH. Oscillatory stress, which is common in curvatures or branching points of the vessel, did not show a similar pattern.

The current understanding of GTPCH regulation in humans and more specifically in patients with coronary artery disease is very limited. This is mainly due to the poor availability of relevant tissues. In some studies, attempts have been made to study neopterin (marker of inflammation) or biopterin levels in the plasma of these patients and link it to cardiovascular events.[28] However, these studies are limited due to the intricate relationship between plasma and vascular biopterins. Antoniades *et al.* [29] described an inverse relationship between plasma BH4 and vascular BH4 in the bypass grafts of patients with coronary artery disease undergoing coronary artery bypass grafts surgery. Patients with high vascular BH4 had bypass grafts with better endothelium-dependent relaxation to acetylcholine than patients with low vascular BH4. High plasma BH4 was paradoxically associated with impaired endothelial-dependent relaxation and low vascular BH4. The authors conclude that plasma BH4 is mainly driven by the contribution of BH4 from inflammatory cells and the liver, whereas vascular BH4 is driven by the rate-limiting enzyme, GTPCH, which is in turn directly correlated to GCH-1 gene expression. As a result, it is not passive diffusion which increases BH4 intracellularly but rather genetic factors which regulate the increase in BH4 in the endothelium, while the loss of vascular BH4 is possibly due to oxidation. What are the consequences of this inverse relationship in patients and how can the induction of endogenous GCH be a possible vascular defence mechanism?

The mechanism of inducing vascular GCH-1 in patients with coronary artery disease was previously not clear and in this thesis we therefore sought to explore this further with a vessel model using coronary artery bypass grafts. This model was then further used to explain some of the findings of two clinical trials (Studies I and II).

Another aspect of GTPCH biology is the possibility for GTPCH degradation via proteasomes. In a study of high glucose stimulation of human endothelial cells, it was reported that GTPCH can be degraded by 26s proteasome.[30] This is an aspect which has not been studied *in vivo* and could potentially also influence BH4 availability.

*1.3.2.3 The salvage pathway and biopterin recycling*

The salvage pathway (Fig 4) refers to the possibility of maintaining BH4 bioavailability through the actions of dihydrofolate reductase (DHFR). This pathway starts with sepiapterin

and, via sepiapterin reductase (SR), BH2 is formed. It can undergo reduction back to BH4 via DHFR. BH2 is able efficiently to replace eNOS-bound BH4, resulting in eNOS uncoupling.[31] Interestingly, in a cell model with the genetic knock-down of DHFR and the inhibition of DHFR with methotrexate, it was observed that the importance of DHFR appears to be more prominent at low levels of GTPCH expression, low levels of BH4 and in conditions of high levels of BH2, which correlates well with what is found in patients with coronary artery disease.[32]

Biopterin recycling refers to the actions of dihydropteridine reductase (DHPR). A deficiency of DHPR is an autosomal recessive condition and has been shown to cause hyperphenylalanemia or phenylketonuria (PKU) due to BH4 deficiency. BH4 supplementation is therefore used in the treatment of PKU. The actions of DHPR with regard to the regulation of eNOS remain to be studied.

### 1.3.3 NADPH oxidase

The family of NADPH oxidase (NOX) consists of seven proteins, where NOX1, NOX2, NOX4 and NOX 5 have been reported to be expressed in the cardiovascular system. They are found in endothelial cells, smooth muscle cells, fibroblasts and phagocytic mononuclear

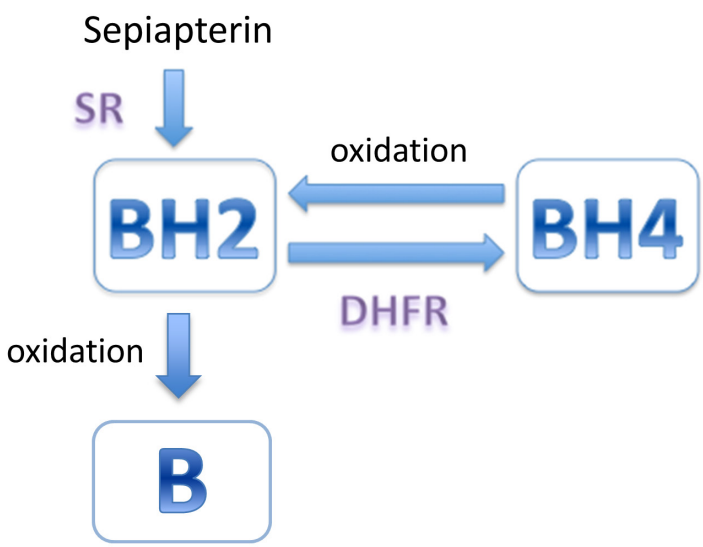


Fig 4. The salvage pathway includes the actions of sepiapterin reductase (SR), which can reduce sepiapterin to dihydrobiopterin (BH2) in all cells, and dihydrofolatereductase (DHFR), which reduces BH2 back to tetrahydrobiopterin (BH4). In a pro-inflammatory setting, BH4 is oxidised to BH2 and, in such a setting, the role of DHFR becomes interesting as a potential mechanism to sustain BH4. The figure also shows the possibility of the oxidation of BH2 to B, which is not part of the salvage pathway but occurs in states of low nitric oxide (NO) availability and increased superoxide production.

cells.[33] NOX 1 and 4 are expressed in vascular smooth muscle cells and NOX2 and 4 are found in endothelial cells.[34] They are transmembrane proteins, which require interaction with a cytosolic activator protein (p47 phox, p67 phox and Rac) in order for ROS generation to occur (Fig 5). This interaction has been carefully studied using inhibitors of Rac and silencing RNA of the cytosolic activator proteins, showing unanimously that these proteins do not produce superoxide without the prior assembly. NOX 4 does not require an activator protein and generates ROS ($H_2O_2$) constitutively. The consequences of NOX 4-dependent ROS are also different in the cell in comparison to the other NOXs, all of which generate superoxide. As a result, there is still a great deal to be explored in terms of the roles of the different NOXs and their interaction.

In coronary artery bypass grafts, Guzik *et al.* were the first to provide evidence that NADPH oxidase is a major contributor of ROS in human vessels and that there were differences in the expression of the different NOXs in the vascular wall.[12] Western blotting and RT-PCR analysis of these vessels revealed that p22 phox and cytoplasmic subunits (p67 phox and p47 phox) were more abundant in saphenous veins than in mammary arteries. In saphenous veins, NOX 2 is mainly found, whereas NOX 4 appears to be more important in mammary arteries. NOX 1 was found in very low levels. Interestingly, cardiovascular risk factors such as hypertension, hypercholesterolemia, obesity and aging are all linked to the increased expression and activity of NOX2.[35]

Sorescu *et al.*[33] were able further to explore the cellular sources of intracellular superoxide production in atherosclerotic and non-atherosclerotic human coronary arteries. They found that all cells are able to produce superoxide, but it is especially high in the shoulder regions of the plaque, which are areas with a high degree of inflammation and risk of rupture. They

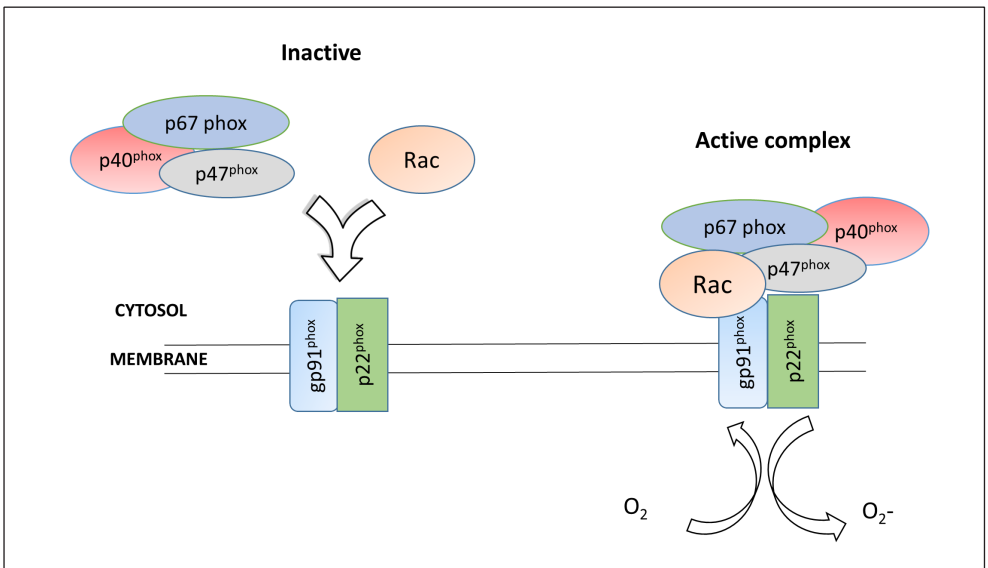


Fig 5. Nicotinamide phosphatase (NADPH) oxidase consists of transmembrane proteins (heterodimer consisting of gp91 phox and p22 phox) which require interaction with a cytosolic activator (p47 phox, p67 phox and Rac1) for the activation and further production of superoxide ($O_2^-$).

also found that superoxide production was low in stable, collagen-rich, macrophage-deficient plaques. Monocytes express NOX 2 mRNA 456 times more than endothelial cells. In coronary arteries, the group found that NOX 2 was mainly expressed in the adventitia, much less in the intimal layer and almost not at all in the medial layer. The severity of atherosclerosis was significantly correlated to NADPH oxidase subunit expression, due mainly to the infiltration of macrophages.[33] In conclusion, patients with coronary artery disease generate ROS dependent on NOX and this is closely related to the presence of inflammatory cells in a pro-atherogenic environment.

### 1.3.4 Other sources of superoxide: Mitochondria and Xanthine oxidase

Superoxide is generated at several sites in the electron-transport system, located on the inner mitochondrial membrane. Mitochondria related superoxide production is closely linked to the energy-generating process of the organelle.[36] The electron transport system involves five large proteins named complex I-V. The leakage of electrons between these complexes is thought to generate superoxide, which is quickly reduced to hydrogen peroxide. Mitochondrial superoxide can also react with NO and form peroxynitrite. The regulation of mitochondrial ROS will not be reviewed here. The contribution of mitochondrial ROS will enhance and stimulate other sources of superoxide, subsequently accelerating the pro-inflammatory process. It is not surprising then that mitochondrial ROS is linked to the activation of pathways such as NF-κB and STAT.

When it comes to xanthine oxidase, it has been shown to reduce molecular oxygen to both superoxide and $H_2O_2$. The inhibition of xanthine oxidase improves vasodilatation in patients with hypercholesterolemia. Due to the lack of sensitive assays which are able accurately to measure xanthine oxidase activity very little is known about xanthine oxidase and its role in endothelial dysfunction.[37]

## 1.4 ENDOTHELINS

Endothelins form a family of peptides consisting of three isoforms, ET-1, 2 and 3. Of these three, ET-1 is linked to endothelial dysfunction and superoxide production.[1] ET-1 is a 21 amino acid long peptide with vasoconstrictive properties. In healthy vessels it is mainly synthesised in endothelial cells from a precursor pre-pro ET-1, which is cleaved into big ET-1 and converted to ET-1 by a group of endothelin-converting enzymes.[38] The peptide has a paracrine/autocrine influence on the vessel. It binds to two G-protein-coupled receptors (GPCR), $ET_A$ and $ET_B$ (Fig 6). $ET_A$ is expressed in vascular smooth muscle cells (VSMC), cardiomyocytes and fibroblasts.[1]

$ET_B$ is found on both VSMC and endothelial cells. The binding of ET-1 to the $ET_A$ receptor leads to vasoconstriction, cell growth, cell proliferation and cell adhesion.[39] Binding to the $ET_B$ receptor on endothelial cells results in the release of NO and vasodilatation. The $ET_B$ receptor also functions as a clearance receptor, via which ET-1 is eliminated from the circulation.[40] NO downregulates the expression and secretion of ET-1. Since ET-1 is immediately secreted and not kept intracellularly, it binds to the $ET_B$ receptor, resulting in NO production via cyclic GMP which in turn reduces the further secretion of ET-1. $ET_B$ receptors are also located on vascular smooth muscle cells and mediate vasoconstriction.

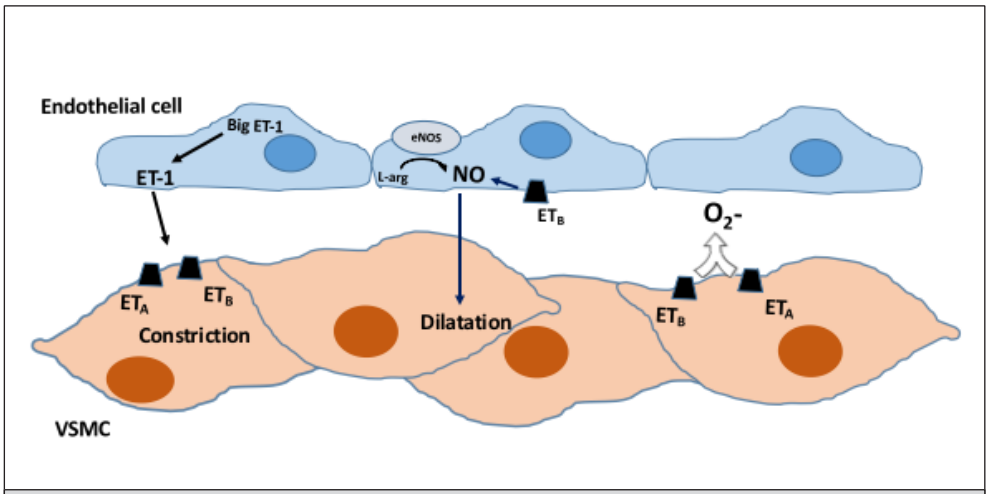


Fig 6. Endothelin-1 (ET-1) is mainly synthesised in endothelial cells in healthy vessels and acts via two G-protein-coupled receptors, $ET_A$ and $ET_B$. These receptors can be found on endothelial cells, vascular smooth muscle cells (VSMC) and adipocytes. The action of ET-1 in a healthy artery results in vasodilatation. In oxidative stress, there is an increase in ET-1 production and a reduction in the bioavailability of nitric oxide (NO), which results in vasoconstriction.

As pointed out below there is a substantial change in the expression and function of the ET receptors in various pathophysiological conditions, resulting in an altered biological response.[41] Increased levels of circulating ET-1 in pathological states such as coronary artery disease or heart failure may be due to the reduced clearance of ET-1 by the $ET_B$ receptor, as well as the increased production of ET-1 in VSMC and inflammatory cells. ET-1 has been shown to increase ROS generation; superoxide, peroxynitrite and $H_2O_2$.

In the following sections, I shall review the mechanisms of ET-mediated superoxide production which will vary depending on tissue and disease state.

### 1.4.1 Mechanisms of ET-mediated superoxide production

It is well known that the influence of ET-1 differs between vascular beds, where larger arteries have a different path of activation in comparison with smaller resistance arteries.[40] This difference also influences the mechanisms behind increased superoxide production. There is a large variety and discrepancy in the findings relating to the source of superoxide, the tissue and the ET receptors that are involved in ET-mediated superoxide production.[42-58]. In Table 1, the most important publications on ET-mediated superoxide production are summarised.

In this section, I shall give a short review of the current knowledge relating to ET-mediated superoxide production and the contribution of NADPH oxidases and eNOS uncoupling. The emphasis will be placed on possible mechanistic explanations of ET-mediated superoxide production.

Table 1. Summary of current scientific articles examining the potential source of ET-mediated superoxide production.

| 1st author | Species | Tissue | Receptor | ROS detection | (In vitro/ In vivo) | Result | Ref |
|---|---|---|---|---|---|---|---|
| Li | Rat | Carotids | $ET_A$ | DHE and Lucigenin CL | In vivo and in-vitro | ET-1 increases superoxide via ETA and NADPH oxidase | 42 |
| Loomis | Rat | Aorta | | Lucigenin CL | In vitro | NADPH oxidase and eNOS uncoupling contributes to ET-1 mediated superoxide. Dual blockade of ET-1 could reduce ET-1 mediated superoxide | 43 |
| Yilmaz | Rat | Thoracic aorta rings | Not studied | None | In vitro | ET-1 mediated vasoconstriction is connected to NADPH oxidase and PARP pathway | 44 |
| Dong | Human | HUVEC | $ET_B$ | CM-$H_2$ DCFDA | In vitro | ET-1 increases $H_2O_2$ via ETB, increases cellproliferation | 45 |
| Zheng | Rat | Carotids | $ET_A$ | Lucigenin CL | In vitro | GTPCH gene transfer increases BH4 and improves vascular redox. | 46 |
| Montezano | Human | EC | Not studied | Lucigenin CL | In vitro | NOX5 is involved in ET-1 mediated superoxide. Independent of Rac1. | 47 |
| Deng | Rabbit | Atrial and ventricular myocytes | $ET_A$ | None | In vitro | Endothelin signalling via NADPH oxidase | 48 |
| Viel | Rat | Aorta and resistance arteries | $ET_A$ | DHE | In vitro | Involvement of $ET_A$ receptor in superoxide generation via xanthine oxidase and mitocondria in aorta and resistance arteries. | 49 |
| Touyz | Human | VSMC | Not studied | CMH2DCFDA fluorescence | In vitro | ET-1 mediates superoxide production via mitochondrial ROS | 50 |
| Dammanahalli | Human | HAAEC | $ET_B$ | DCFH-DA, DHE | In vitro | ET-1 increases superoxide NADPH oxidase: via ETB- Pyk2-Rac1-Nox1 Pathway | 51 |
| Romero | Rat | Aortic rings | $ET_A$ | DHE | In vitro | ET-1 increases superoxide via NADPH oxidase, increase of p47 phox expression, involving c-Src activation and ERK ½ phosphorylation | 52 |
| Duerrschmidt | Human | HUVEC | $ET_B$ | Coelenterazine CL | In vitro | ET-1 increases superoxide via ET-B-NADPHoxidase (gp91 phox) | 53 |
| Sarkar | Bovine | PASMC | $ET_A$ | SOD inhibitable cytochrome c reduction assay | In vitro | ET-1 increases superoxide via ETA-NADPH oxidase and induces an increase in proMMP-2activation via NADPH oxidase-PKC-p38MAPK-NF-KB signaling. | 54 |
| Sanchez | Rat | Penile arteries | $ET_A$ | Lucigenin CL | In vitro | ET-1 increases superoxide via ETA- NADPH oxidase | 55 |
| Ergul | Human | Saphenous vein | Not studied | DHE and lucigenin CL | In vitro | ET-1 increase superoxide in SV but not via NADPH oxidase | 56 |
| Meyer | Mouse | Carotid artery | Not studied | None | In vitro | Vascular responses to ET-1 are NADPH oxidase dependent and mediated by inducible NOX-1/ and or NOX 2 isoforms, incl increased expression of p47 phox. | 57 |
| Callera | Rat | Aorta, mesenteric arteries and heart | $ET_A$ | Lucigenin CL | In vitro | ET-1 mediates increased superoxide independent of NADPH oxidase, nor Xanthine oxidase or eNOS uncoupling. Possible role for mitochondrial ROS | 58 |

*1.4.1.1 NADPH oxidase*

To date, there are four main mechanistic possibilities describing the role of NADPH oxidase in ET-mediated superoxide production.

(1) The correlation between ET-mediated vasoconstriction and NADPH oxidase activity[43 57]

(2) The increased expression of ET receptors and the ratio of $ET_A$ to $ET_B$ [57 59]

(3) The increased expression of NADPH oxidase subunits in the vasculature in relation to ET-1[52]

(4) Activation of down-stream signaling of G-protein coupled receptors such as mitogen activated protein kinase (MAPK)[54] ERK 1/2[52] pathways, poly ADP ribose polymerase (PARP) pathway[44] and Pyk2-Rac1-Nox1-pathway[39 51].

The link between ET-1 contractility and NADPH oxidase was shown in a mouse model with the aim of studying the role of aging.[57] Aging increased ET-1 contractility, ET-receptor expression with a switch towards increased $ET_B$ and the increased expression of the NADPH oxidase subunit, p47 phox. The authors conclude that vascular responses to ET-1 are NADPH-oxidase dependent and are mediated by the inducible NOX1 and/or NOX2 isoforms, but also increased vascular p47phox gene expression may explain the increase in NADPH oxidase activity induced by ET-1.

In atherosclerotic vessels, several studies have demonstrated that an increase in $ET_B$ to $ET_A$ receptor expression occurs, as well as an increase in the levels of big ET-1 and endothelin-converting enzyme, indicating an increase in the synthesis of ET-1.[59] It has also been shown that it is possible that inflammatory cells are able to modulate the switching of ET receptor subtypes from $ET_A$ to $ET_B$ in vascular smooth muscle cells. In human atherosclerotic vessels, there was an increase in the immunoreactivity of ET-1 and $ET_B$ receptors in both non-foamy and foamy macrophages, T lymphocytes in fatty streaks and fibrous plaque lesions. In addition, medial SMCs located just beneath the foam cell lesions revealed a higher intensity of $ET_B$ receptor immunoreactivity than those located beneath the normal-looking intima without foam cells. In fibrous plaques, intimal smooth muscle cells near foam cells showed an increase in the density of ET receptors with predominant $ET_B$ immunoreactivity. In the areas where vascular smooth muscle cells expressed an increased density of $ET_B$ receptors, ET-1 immunoreactivity was also enhanced.[60] These findings suggest that oxidative stress may be mediated by both $ET_A$ and $ET_B$ receptors on VSMCs in human atherosclerosis.

Regarding GPCR signaling, a recent study by Sarkar *et al.* describes a model in pulmonary artery smooth muscle cells which highlights a few interesting mechanistic pathways involved in ET-mediated superoxide production. In this model, ET-1 increases superoxide via the $ET_A$ receptor involving NADPH oxidase and signaling through the protein kinase C (PKC)->MAPK->NF-Kβ pathway.[54]

Romero *et al.*[52] studied ERK1/2 pathways in rat aortic rings and they were able to show that ET-1 increases superoxide production via an increase in p47 phox expression involving a non-receptor tyrosine protein kinase (c-Src) and ERK1/2 phosphorylation.

A recent intracellular downstream signaling pathway connected to oxidative stress in relation to atherosclerosis is poly (ADP ribose) polymerase (PARP-1). In thoracic rings from rat, ET-1 induced endothelial dysfunction which could be restored after incubation with PEG-SOD (superoxide scavenger), an inhibitor of NADPH oxidase (apocynin) and a PARP inhibitor. In addition, Western blots on vessel rings which had been stimulated with ET-1 showed an increase in the expression of PARP.[44] Collectively, these findings suggest that ET-1 may contribute to oxidative stress through NOX in a variety of different tissues.

*1.4.1.2 eNOS uncoupling*

To date, very few studies have been able to show a link between eNOS uncoupling and ET-mediated superoxide production. Of these, only three studies[43 46 61] are related to ET-mediated superoxide production and the possible involvement of eNOS uncoupling.

Loomis *et al.*[43] and Zheng *et al.*[46] have explored this possible link to some degree. Loomis *et al.* found that adding BH4 to rat aortic rings incubated with ET-1 led to reduced superoxide production and improved relaxation responses to acetylcholine, indicating an improvement in endothelial function.

Zheng *et al.*[46] reported that the gene transfer of GTPCH restored arterial GTPCH activity and BH4 levels, resulting in reduced superoxide and improved endothelium-dependent relaxation and basal NO release in deoxycorticostereone (DOCA)-salt rats. The authors concluded that BH4 deficiency resulting from ET-induced superoxide via an $ET_A$/NADPH oxidase pathway leads to endothelial dysfunction, while the gene transfer of GTPCH I reverses BH4 deficiency and endothelial dysfunction by reducing superoxide in low renin mineralocorticoid hypertension. However, BH4 may have acted as an anti-oxidant in these studies, thereby reducing superoxide production and improving endothelial function. Direct effects on eNOS coupling were not studied.

Furthermore, the direct effect of ET-1 on BH4 is unknown. Romero *et al*.[61] studied the influence of quercetin (a flavonol found in fruit and grains) on ET-mediated superoxide production due to NADPH oxidase and eNOS uncoupling. In aortic rings, ET-1 impaired vasorelaxation to acetylcholine which can be restored with an inhibitor of NADPH oxidase (apocynin), partially by superoxide dismutase (SOD) but not sepiapterin. ET-1 significantly increased superoxide as measured by lucigenin-enhanced chemiluminescence in aortic rings, which could be inhibited with apocynin and sepiapterin. The authors concluded that ET-mediated superoxide production originates from NADPH oxidase and eNOS uncoupling. It is, however, evident that adding sepiapterin did not improve endothelial function and it is therefore not clear whether eNOS uncoupling is a causal explanation of ROS-induced endothelial dysfunction in the case of ET-1.

The very few studies and the lack of conclusive data give an indication of the need for more studies to explore the influence of ET-1 on eNOS uncoupling.

## 1.5 SUMMARY

Endothelial dysfunction is a critical step in the development of atherosclerosis. Oxidative stress plays an important role in reducing the bioavailability of NO through direct oxidation and through decreased production from eNOS. The generation of excessive amounts of ROS leads to not only reduced NO but also oxidative modifications of key proteins which can initiate and sustain disease progression. Sources of ROS are eNOS uncoupling, NADPH oxidase, mitochondria and xanthine oxidase.

In this thesis, I have sought to explore further the regulation of eNOS in coronary artery disease using tissue from patients undergoing coronary artery bypass surgery. In this tissue, NADPH oxidase and eNOS uncoupling are the most important sources of ROS. eNOS is closely regulated by its essential co-factor, BH4, and, more importantly, the ratio of BH4:BH2+B. The availability of BH4 is influenced by oxidative processes of the cell but also by synthesis and salvage pathways.

In the pro-atherogenic environment, ET-1, a vasoconstrictor, is increased and influences NO bioavailability through the generation of ROS. ET-1 acts through its receptors with an intricate downstream signaling system. Whether ET-1 influences NADPH oxidase and eNOS uncoupling is not fully clear and we have sought to study this further.

# 2 HYPOTHESIS AND AIMS

The main hypothesis of this thesis was that eNOS and endothelial function is regulated by the availability of BH4.

Four studies were designed with the specific aim of investigating:

(1) Exogenous BH4 and effects on endothelial function in patients with coronary artery disease (Study I)

(2) The regulation of endogenous BH4 and subsequent effects on endothelial function in patients with coronary artery disease (Study II)

(3) The source of ET-mediated superoxide production and the involvement of an ET-receptor-dependent mechanism in patients with coronary artery disease (Study III)

(4) BH4 availability during increased ET-mediated superoxide production and effects on endothelial function (Study IV)

# 3 MATERIALS AND METHODS

## 3.1 STUDY SUBJECTS

All the investigations were carried out in accordance with the Declaration of Helsinki and were approved by the regional ethics committee at Karolinska Institutet or Oxford University. Study subjects were patients admitted to the Cardiothoracic Unit at John Radcliffe Hospital, Oxford, UK, awaiting coronary artery bypass surgery. Recruitment took place at the hospital one day before surgery except in the clinical trial (study I). The patients were informed about the purpose of the study and the possible risks of participating. All patients gave their written consent to take part in the studies. Baseline characteristics for all studies are shown in Table 2. In Study 4, endothelial function is assessed in resistance arteries from six healthy women undergoing elective caesarean section at Karolinska University Hospital, Sweden.

### 3.1.1 Study I

A total of 55 patients agreed to donate tissue from surgery. Of these, 49 patients took part in the clinical trial and the remaining six patients were only recruited for the *ex vivo* vessel experiments.

### 3.1.2 Study II

A total of 445 patients and 20 healthy subjects were included in the study. Of these, 19 patients were recruited for the *ex vivo* vessel experiments.

### 3.1.3 Study III

We recruited 90 patients. The inclusion criteria were coronary artery disease in need of elective or subacute coronary artery bypass surgery and the exclusion criteria were emergency CABG and unwillingness to participate.

### 3.1.4 Study IV

We recruited 41 patients. The inclusion criteria were elective and subacute coronary artery bypass grafts surgery and the exclusion criteria were emergency bypass surgery and unwillingness to participate.

## 3.2 CLINICAL ASSESSMENT OF VASCULAR FUNCTION

### 3.2.1 Brachial flow-mediated dilation and magnetic resonance imaging

Flow-mediated dilation (FMD) is the method that is most widely used for measuring endothelial function and specifically the ability of arteries to respond to endothelial NO release during reactive hyperaemia (flow mediated).[62] A blood pressure cuff on the forearm is used to occlude the flow in the brachial artery for five minutes. When the pressure of the cuff is released, the arterial flow increases, giving rise to increased NO release from the endothelium. The difference in the diameter of the brachial artery before and after occlusion is then measured using ultrasound (Study II) or, in the case of Study I, magnetic resonance imaging. In order to measure endothelium-independent dilation, subjects received 200 µg of glyceryl trinitrate sublingually. Endothelium-independent dilation was calculated as

the percentage increase between the baseline luminal area and the maximum luminal area following the administration of glyceryl trinitrate.

Leeson *et al*.[63] reported good agreement and reproducibility between the two modalities and here is a summary of the advantages of each modality:

The advantages of MRI are:

(1) Full three-dimensional visualisation of the vessel, enabling the imaging plane to be placed perpendicular to the vessel in a reproducible location

(2) The ability to measure other parameters of vascular structure and function (aortic and carotid distensibility) as part of the same examination.

The advantages of ultrasound are:

(1) A higher temporal resolution than MRI, allowing more precise determination of the 'peak of the curve' of dilation

(2) Lower cost

(3) Better subject tolerability (no claustrophobia).

Table 2. Baseline characteristics of study subjects from Studies I-IV

| | **Study I** | | | | **Study II** | | | | | | | | **Study III** | **Study IV** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Placebo | 400 mg/d | 700 mg/d | Ex vivo | **Part I** | | **Part 2** | | | **Part 3** | | **Part 4** | | |
| | | | | | Vacc | Placebo | OO | XO | XX | OO | XX | Ex vivo | | |
| Subjects (n) | 19 | 14 | 16 | 6 | 10 | 10 | 280 | 85 | 11 | 40 | 10 | 19 | 90 | 41 |
| Age (SEM) | 68 | 69 | 68 | 71 | 29 | 31 | 65 | 67 | 67 | 60 | 66 | 68 | 68 | 68 |
| ***Risk factors*** | | | | | | | | | | | | | | |
| Diabetes mellitus (%) | 26 | 36 | 25 | 17 | 0 | 0 | 29 | 26 | 23 | 23 | 20 | 10 | 28 | 27 |
| Hypertension (%) | 68 | 79 | 69 | 50 | 0 | 0 | 68 | 78 | 76 | 72 | 80 | 68 | 70 | 63 |
| Smokers (%) | 11 | 14 | 25 | 17 | 30 | 40 | 40 | 33 | 38 | 51 | 48 | 26 | 16 | 12 |
| BMI (SEM) | 27 | 30 | 27 | 30 | 25 | 25 | 28 | 28 | 28 | 29 | 27 | 28 | 28 | 29 |
| ***Medication*** | | | | | | | | | | | | | | |
| Aspirin (%) | 100 | 79 | 88 | 67 | 0 | 0 | 77 | 80 | 85 | 80 | 80 | 63 | 79 | 51 |
| Statin (%) | 95 | 93 | 100 | 83 | 0 | 0 | 82 | 82 | 80 | 26 | 90 | 78 | 88 | 95 |
| ACEi and/or ARB (%) | 84 | 79 | 56 | 50 | 0 | 0 | 68 | 67 | 67 | 74 | 60 | 72 | 59 | 81 |
| B blocker (%) | 84 | 71 | 56 | 83 | 0 | 0 | 77 | 75 | 65 | 76 | 70 | 73 | 70 | 85 |

The raw data yielded by ultrasound and MRI are not the same. Ultrasound measures changes in vessel diameter in the longitudinal plane, whereas MRI measures changes in vessel cross-sectional area. The FMD value measured by ultrasound will translate to a higher FMD value as compared to measured by MRI, as vessel area is proportionate to the square of vessel radius.[63]

A detailed description of the MRI protocol can be found in the published article relating to Study I.

### 3.2.2 Arterial stiffness

Local NO bioavailability regulates arterial elasticity in humans *in vivo*. Arterial stiffness is correlated to cardiovascular risk.[64] MRI can be used to measure arterial stiffness. Blood flow data can be acquired simultaneously and the path length (the distance between the two aortic locations) can be measured precisely. In Study I, MRI was used to measure arterial stiffness (aortic/carotid distensibility and aortic pulse wave velocity).

All the above-mentioned methods for assessing vascular function *in vivo* were performed by collaborators in Oxford, UK.

## 3.3 CORONARY ARTERY BYPASS GRAFTS

### 3.3.1 Handling and dissection

Internal mammary artery (IMA) and human saphenous vein (SV) segments were collected from the surgical theatre at John Radcliffe Hospital, Oxford, UK. The segments were immediately gently flushed with oxygenated ice-cold Krebs-Henseleit buffer and then immersed in the buffer while awaiting transport to the lab. Perivascular fat and connective tissue surrounding the vessel were gently removed with surgical tools. The vessels were then immediately used for vascular superoxide measurement, biopterin measurement, vasomotor studies or *ex vivo* incubation.

### 3.3.2 Protocol *ex vivo* experiments

The model for *ex vivo* experiments was developed using vein and mammary grafts from an additional 30 patients. These initial experiments laid the foundation for a protocol which could then be used in Studies I and II. Because of the nature of biopterins, conditions must be such that the external influence of the oxidation of BH4 and endotoxin or bacterial infiltration, for example, is not present. The very first experiments in somewhat unsterile conditions revealed a huge increase in BH4 overnight (24 h), indicating the presence of bacteria or fungi. For this reason, all further experiments were performed under sterile and endotoxin-free conditions.

Vessel segments were immersed in oxygenated cold Hanks Buffered Saline Solution (HBSS, consisting of sodium bicarbonate without calcium chloride and magnesium sulphate) buffer with 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid (HEPES) in theatre, after which the vessel segments were gently flushed. Within 15 minutes, the segments were transferred to the laboratory. The vessels were dissected and cut into 3 mm thick rings. One ring from each

patient was immediately snap frozen and labelled time 0. The remaining vessel rings were placed in six-well cell-culture plates with sterile HBSS containing HEPES, 1% penicillin streptomycin and 1% amphotericin B and further placed in a cell incubator with 95% oxygen ($O_2$) and 5% carbon dioxide ($CO_2$). Concentrations of antibiotics and anti-fungi were carefully determined in a set of experiments in order to ensure that they did not interfere with biopterin homeostasis. The use of HBSS was optimal, as normal endothelial cell media usually contain serum and other additives which can interfere (as seen on the read-out of biopterin levels).

The vessels were incubated in a cell incubator and checked macroscopically after 24 h for signs of infection. They were then snap frozen and kept at -80°C until analysed for biopterin measurements, gene expression and protein. In order to make sure that the conditions under which the vessels were kept did not affect endothelial function, a few vessels were transferred to an organ bath to evaluate endothelial function by quantifying the relaxation response to ACH at time 0 vs 24 h. It then became clear that the vessels that had been kept overnight had an increased response to ACH in comparison to time 0. So, in a first series of experiments, I sought to explain this finding and link it to the substantial increase in BH4; whether it was endothelial specific, whether it was driven by synthesis or salvage pathway, as possible mechanistic explanations.

In order to study whether the increase in BH4 was endothelial specific, a few vessel rings underwent endothelial denudation and were then stored at -80°C for subsequent analysis. The question of synthesis vs salvage pathway could be studied using an inhibitor of GTPCH, diamino-6-pyrimidine (DAHP), and an inhibitor of DHFR, methotrexate. When the model appeared to deliver robust data, it could then be used to answer more specific questions within the clinical trials (Study I + II).

*3.3.2.1 Incubation schedule Study I*

Vessel segments from six patients were incubated for 30 min in oxygenated Krebs Hepes Buffer at 37°C either in buffer alone or in the presence of BH4 (100 nmol/L, Schirks Laboratories, Jona, Switzerland), BH4 plus the antioxidant, dithioerythritol (DTE, 1 mmol/L) or BH2 (100 nmol/L, Schirks Laboratories). Samples of incubation media and vessel rings were stored at -80°C prior to the quantification of BH4, BH2 and biopterin by high-performance liquid chromatography (HPLC) with electrochemical detection for BH4 and fluorescent for BH2 and B.

*3.3.2.2 Incubation schedule Study II*

Four sequential rings of SV and IMA from the same patient were incubated for 24 h *ex vivo* in the absence (control) or presence of the GTPCH inhibitor, DAHP (1 mmol/L), with or without stimulation by an inflammatory cytokine cocktail consisting of TNFα (4 ng/mL) plus IL-6 (25 ng/mL) plus LPS (80 ng/mL). After incubation, vascular rings (SV only) were transferred to an organ bath to evaluate endothelial function by quantifying the vasomotor responses to acetylcholine ACH and the endothelium-independent vasodilator, sodium nitroprusside (SNP). Some vessel rings were kept at -80°C for the quantification of BH4, BH2 and biopterin and GCH mRNA expression.

### 3.3.3 Organ bath

Endothelium-dependent and -independent dilations were assessed with isometric tension studies. Vessel rings were equilibrated and passively pre-tensioned to 3 g, an optimal resting tension that was determined in baseline studies of the contractile response to potassium chloride (KCl). After precontraction with phenylephrine ($3x10^{-6}$ mol/L), vasomotor responses to the endothelium-dependent vasodilator, ACH ($10^{-9}$ to $10^{-5}$ mol/L), were quantified in the four equally sized segments from the same vessel, as described in the previous paragraph. Finally, relaxations to the NO donor, sodium nitroprusside (SNP, $10^{-10}$ to $10^{-6}$ mol/L), were evaluated in the presence of the NOS inhibitor, $N^G$-nitro-L-arginine methyl ester (L-NAME 100 μmol/L).

### 3.3.4 Vascular superoxide measurement

The quantification of reactive oxygen species in vascular tissue is difficult for the following reasons.

(1) Superoxide is generated in very small amounts due to instant reactions with surrounding molecules and very effective scavenging by for example superoxide dismutases and NO.

(2) Some probes are able to generate superoxide themselves due to redox cycling and

(3) The inability of most assays accurately to quantify the generation of intracellular superoxide.[65]

Among the large variety of assays lucigenin-enhanced chemiluminescence and dihydroethidium (DHE) have been proven to have high enough sensitivity and specificity for detection of superoxide in vascular tissue.[66]

#### *3.3.4.1 Lucigenin-enhanced chemiluminescence*

This method uses the cell-permeable chemiluminescent probe, bis-N-methylacridinium, or lucigenin chemiluminescence, which releases a photon when it is exposed to superoxide. This emission of light can be detected by a luminometer. Briefly, superoxide reduces lucigenin to its cation radical, which reacts with a second superoxide molecule to form dioxetane emitting a photon.[66] This method has been used extensively as an indicator of intracellular superoxide in many different vessel models (for references, please see Table 1). The method has been challenged due to the fact that it can react with oxygen when there are high concentrations of lucigenin, creating a system for redox cycling originating from the tissue itself. At low concentrations (<5 μmol/L) of lucigenin, this type of redox cycling is not seen. This has been proven with electron paramagnetic resonance.[67]

Lucigenin-enhanced chemiluminescence was used to measure vascular superoxide in paired segments of SV and IMA in Studies III and IV. The segments were divided into four to six rings, each approximately 3 mm thick, depending on size, weight ranging from 6-20 mg/ring. The vessel segments were opened longitudinally to expose the endothelial surface and equilibrated in oxygenated (95% $O_2$/5% $CO_2$) Krebs-HEPES buffer (pH 7.4) at 37°C. As a measure of eNOS coupling, we determined NOS-derived production in paired segments, which was estimated as the difference in superoxide production after 20 min of incubation with the NOS inhibitor, L-NAME, alone [68] or followed by incubation with ET-1 (0.1 nM) for

45 min. The vessel segment was then quickly transferred to a luminometer containing low-concentration lucigenin (5 μM) in order to measure superoxide production. In a separate set of experiments, additional rings were analysed after 20 minutes' pre-incubation with the $ET_A$ receptor antagonist, BQ123 (1 μM), alone, or in combination with the $ET_B$ receptor antagonist, BQ788 (1 μM; dual BQ), the NADPH oxidase inhibitor, apocynin, the mitochondrial ROS inhibitor, rotenone, and the xanthine oxidase inhibitor, oxypurinol, followed by ET-1 (0.1 nM) for 45 min. At the end of these experiments, the NADPH-stimulated superoxide production was estimated 10 min after NADPH (0.1 mM) was added, as previously described [68]

*3.3.4.2 Dihydroethidium*

The oxidative fluorescent probe, DHE, is used in detecting superoxide in intact tissues. Its lipophilic nature makes the permeation of cell membranes possible. After reacting with superoxide (forming 2OH-$E^+$), this blue fluorescent probe will oxidise to ethidium bromide ($E^+$) and bind to DNA, which stains the cell nucleus red. The analysis of the tissue and the fluorescence is measured with confocal microscopy.[69] The reaction forming 2OH-$E^+$ can also occur via other pathways involving cytochrome P450 and this method is therefore a qualitative rather than a quantitative indicator of superoxide.[66]

*In situ* superoxide production was determined in vessel cryosections with the oxidative fluorescent dye, DHE, as previously described [70]. Paired vessel segments were incubated in the presence or absence of ET-1 (0.1nM) in Krebs-HEPES buffer for 45 min at 37°C and then snap frozen in tissue tek optimum cutting temperature (OCT) liquid in preparation for further cryosections. Cryosections (30 μm) were equilibrated in the presence or absence of ET-1 for 30 min at 37°C and then exposed to DHE (2 μM) for five minutes. Fluorescence images of the endothelium (x63, Zeiss LSM 510 META laser scanning confocal microscope) were obtained from each vessel quadrant. Segments of vessel rings (with and without ET-1) were analysed in parallel with identical imaging parameters. DHE fluorescence was quantified by automated image analysis with Image-Pro Plus software (Media Cybernetics, Bethesda, Md, USA).

### 3.3.5 Quantification of biopterins

BH4, BH2 and biopterin (B) levels in vessel tissue lysates, homogenates from the lungs and aortas of transgenic (ET-TG) mice with ET-1 overexpression in the endothelium, [71], cell lysates, and plasma lysates were determined by HPLC, followed by electrochemical (for BH4) and fluorescent (for BH2 and B) detection. All HPLC measurements were performed at the Channon Laboratory at the Department of Cardiovascular Medicine, University of Oxford, where the method has been extensively validated for several years [16]. Samples were injected onto an isocratic HPLC system and quantified using sequential electrochemical (Coulochem III, ESA Inc., UK) and fluorescence (Jasco, UK) detection. HPLC separation was performed using a 250 mm, ACE C-18 column (Hichrom, UK) and mobile phase, comprising 50 mM sodium acetate, 5 mM citric acid, 48 μM ethylene diamine tetra-acetic acid (EDTA) and 160 μM DTE (pH=5.2) (all ultrapure electrochemical HPLC grade) at a flow rate of 1.3 ml/min.

BH4 was measured directly by the electrochemical detector (background currents of +500 nA and -50 nA were used for the detection of BH4 on electrochemical cells E1 and E2 respectively). 7,8-BH2 and biopterin were measured as separate chromatographic peaks,

in the same sample, using a Jasco FP2020 fluorescence detector, serially connected to the electrochemical detector. The quantification of BH4, BH2 and B was performed by comparison with external standards. All the samples were loaded in the HPLC auto-sampler in random batches (of up to 12 samples at a time). Each batch was run with an individual standard curve for BH4, BH2 and B. Biopterin levels were expressed as pmol/mg of tissue or protein.

### 3.3.6 Quantification of GTPCH activity

GTPCH activity was measured by HPLC analysis after iodine oxidation. Samples were incubated with GTP to produce 7,8 dihydroneopterin triphosphate. Oxidation by potassium iodide and iodine forms neopterin triphosphate and dephosphorylation by alkaline phosphate produces neopterin, which can be detected by HPLC fluorescent detection. Snap-frozen tissue samples (30 mg) underwent three freeze-thaw cycles and cell pellets were freeze thawed in lysis buffer (0.1 M Tris, 0.3 M KCl, 2.5 mM EDTA and 100 µM phenylmethylsulfonyl fluoride pH 7.8). 250 µl of tissue and cell lysates were incubated for one hour at 37°C with 50 µl of 10 mM GTP in the absence of light. Samples were then oxidised with 10 µl of potassium iodide/iodine 0.1 M and deproteinated with 10 µl 1 M hydrochloric acid for one hour at room temperature in the absence of light. The reaction was stopped by the addition of 10 µl 0.1 M ascorbic acid. 1 M sodium hydroxide and 16 U/ml of alkaline phosphatase were added for one hour at 37°C in the absence of light. Samples were injected onto an isocratic HPLC system and quantified using fluorescence (JASCO, UK) detection. HPLC separation was performed using a 250 mm, ACE C-18 column (Hichrom, UK) and mobile phase comprising 50 mM sodium acetate, 5 mM citric acid, 48 µM EDTA and 160 µM DTE (pH 5.2) at a flow rate of 1.3 ml/min. All the chemicals that were used were ultra-pure HPLC grade. The quantification of neopterin was carried out by comparison with external standards and normalised for sample protein content. Subsequently, the protein concentration of each sample was measured using the BCA Protein Assay kit (Pierce, USA).

### 3.3.7 Reverse transcription polymerase chain reaction

Segments of vessels were immersed in trizol before they were snap frozen. Trizol is a phenol-based liquid which maintains ribonucleic acid (RNA) integrity during homogenisation. The segments were then thawed and 500 µl of trizol was added to each vessel segment, after which they were homogenised for one minute on ice. To each homogenate, 200 µl of chloroform was added. The samples were shaken vigorously for at least two minutes. They were left at room temperature and then centrifuged at 13K for 15 min at 4°C. The aqueous phase was removed and 700 µl of 70% ethanol was added. The samples were subsequently added to RNeasy columns (Qiagen, UK), where total RNA binds to the membrane of the column and contaminants are washed away. Two different RNeasy kits (Qiagen, UK) were used, depending on sample size; for samples of < 5 mg, an RNeasy micro kit was used, while RNeasy mini kits were used for larger samples. Qiagen provides a protocol for RNeasy kits which was followed. RNA was converted into cDNA (superscript II reverse transcriptase, Invitrogen), then subjected to a quantitative real-time polymerase chain reaction using the TaqMan system (Applied Biosystems; assay ID GCH Hs00609198-m1, assay ID GAPDH Hs02758991-g1, assay ID eNOS Hs01574659-m1) and analysed on an Icycler IQ (Bio-Rad). Relative expression was calculated using the $2^{-\Delta\Delta CT}$ method (normalised to endogenous control GAPDH).

### 3.3.8 Western blotting

Protein was extracted from frozen segments of SV and IMA with lysis buffer (50 mmol/L Tris, pH 7.5, 150 mmol/L NaCl, 0.1% sodium dodecyl sulphate, 0.5% deoxycholate, 1% Nonidet P-40) containing protease inhibitors (Complete; Roche) and 1 mmol/L phenylmethylsulfonyl fluoride. Protein lysates (5 to 15 µg) were separated by electrophoresis on 4% to 12% NuPAGE Bis-Tris gels (Invitrogen, Carlsbad, Calif). Rabbit anti-human GTPCH (1:2000) was kindly provided by Gabriele Werner-Felmayer, Inst. for Medical Chemistry and Biochemistry, Austria. eNOS and GAPDH were detected using mouse monoclonal antibodies (BD Biosciences, San Jose, Calif, and Chemicon International, Temecula, Calif, respectively). Mouse anti-rabbit glyceraldehyde 3-phosphate dehydrogenase (GAPDH, 1:5000, Millipore, USA) and mouse anti-bovine α-actinin (1:2000, Sigma Aldrich Inc) were used to confirm equal protein loading. Protein bands were visualised by chemiluminescence (Supersignal Pierce, USA).

### 3.3.9 Determination of protein content

Sample protein content was measured using the BCA™ protein assay kit (Pierce, USA). Standards were prepared from bovine serum albumin (BSA, 2 mg/ml stock, Sigma Aldrich Inc). Standards, samples and an assay buffer blank (12 µl) were mixed with a 20 mg/ml solution of iodoacetamide (12 µl) (Sigma Aldrich, Inc) and incubated at 37°C for 15 min. Reagent A and B were mixed 50:1 respectively and 480 µl was added to all standards, samples and blank. Tubes were incubated for 30 min at 37°C. Standards, samples and blank (2 x 240 µl) were added to a 96-well plate and read at 562 nm. The concentration of protein within each sample was calculated using the standard curve.

## 3.4 RESISTANCE ARTERIES FROM HEALTHY SUBJECTS

*3.4.1.1 Study subjects, handling and test of vasomotor responses*

Healthy women undergoing planned elective caesarean section were asked to donate subcutaneous fat. All the women gave their oral and written consent. A subcutaneous fat sample (dimensions: L, <50 mm; W, <20 mm; T, <10 mm) was collected in ice-cold Physiological Salt Solution (PSS; composition, see below), repeatedly rinsed with ice-cold PSS and stored for the remainder of the procedures on ice. A small piece of the biopsy (20 x 20 mm) was then pinned on a silicone substrate in ice-cold PSS (exchanged every 10 min) and subcutaneous, resistance arteries (RA) were isolated by micro-dissection. After the RA were trimmed of perivascular fat and connective tissue, two wires (diameter 0.04, stainless steel) or 0.025 mm (tungsten) were carefully inserted into the lumen. The resistance artery with the wires was then transferred to one of the wells of a four-channel myograph (Danish Myo Technology, www.dmt.dk) and mounted on specimen holders to record transverse isometric force, as previously described.[72]

## 3.5 ANIMAL MODELS

In this thesis, we present data from a mouse model with the overexpression of human preproET-1 specifically in the endothelium, the ET-TG mouse.[71] The mouse has three times higher vascular tissue ET-1 mRNA and seven times higher ET-1 plasma levels in comparison

with WT, indicating that the increase in gene expression was also linked to the increased secretion of ET-1. There were no significant differences in blood pressure or heart rate between ET-TG and WT mice. Resistance arteries from the ET-TG mouse have a significantly reduced relaxation response to ACH and a blunted contraction response to ET-1 in comparison with WT mice. No difference in contraction to norepinephrine was seen. $ET_B$ receptor mRNA and protein were significantly increased in comparison with WT. With regard to superoxide production, NADPH oxidase activity was measured with lucigenin CL, showing significantly increased activity in comparison with WT. Moreover, the NADPH oxidase subunit gp91 phox protein was increased, indicating a link between ET-mediated superoxide production and NADPH oxidase.[71] Resistance arteries were also tested for relaxation responses to ACH in the presence and absence of L-NAME, vitamin C and Tiron in the original publication. The ET-TG arteries showed an improvement in relaxation responses in the presence of vitamin C, indicating a role for ROS and, with L-NAME, there was an inhibitory response but still slightly better than in WT, indicating that there might be a small possibility of eNOS uncoupling.

We had the opportunity to quantify biopterins in the aorta, lung and plasma from the ET-TG mouse. We used the same protocol as described in 3.3.5. We did not study the resistance arteries, as these vessels are so small that quantifying biopterins becomes near impossible.

## 3.6 ENDOTHELIAL CELLS

### 3.6.1 sEnd.1 cells and HUVEC

sEnd.1 cells are mouse endothelioma cells[73] and they have a substantially increased expression of GCH and an abundance of BH4. We quantified BH4, BH2 and biopterin in the presence and absence of ET-1. They were also used as positive controls for GTPCH activity assays and Western blots. Human umbilical vein endothelial cells (HUVEC) were purchased from Lonza and grown to confluence in a flask with endothelial cell-specific medium. After three passages, the cells were used to measure biopterins in the presence and absence of ET-1.

## 3.7 STATISTICAL ANALYSIS

The results are presented as the mean and standard error of the mean (SEM), apart from Study III, where all the data are the mean and standard deviation (SD). All variables were tested for normal distribution using the Kolmogorov-Smirnov test. Non-normally distributed variables were log transformed for analysis. For the *ex vivo* experiments in Studies I and II, one- or two-factor ANOVA was used, followed by the Bonferroni *post*-hoc test to compare variables between groups. For the vasomotor studies, vasorelaxation curves were compared by two-way ANOVA for repeated measures. All the tests were two-tailed and values of $p < 0.05$ were considered significant. All the statistical tests were performed with either SPSS (Studies I and II) or Graph Pad prism (Studies III and IV).

# 4 RESULTS

## 4.1 ENDOGENOUS BH4 AND ITS REGULATION

### 4.1.1 Model for *ex vivo* incubations of SV and IMA

A model for *ex vivo* incubations was created for SV and IMA (unpublished data, for details: methods section 3.3.2). This model was further used in Study I and Study II. The incubation of SV overnight (24 h) without any stimuli resulted in a significant increase in BH4 (Fig. 7A; endothelialised SV: n=24, $p<0.0001$, endothelium de-nuded vessels: n=5) but not in BH2 (Fig. 7B, SV n=24; $p>0.05$, endothelium de-nuded vessels: n=5). This increase in BH4 is linked to an increase in the activity of the rate-limiting enzyme, GTPCH (Fig. 7E, SV with endothelium, n=11, $p<0.0001$). It is also linked to an increase in the mRNA expression of GCH (Fig 7F, SV, n=6) and GTPCH protein expression (Fig 7H, SV, representative figure of n=2). eNOS protein expression was also increased (Fig 7H, SV, representative figure of n=2).

Further, a subset of vessels (Fig 7C-D, n=3 of SV and IMA) were incubated with the GTPCH inhibitor, DAHP, and the DHFR inhibitor, methotrexate. In this small set of experiments, there appears to be an indication (although not significant due to the small-scale observation) that the increase in BH4 may be related to synthesis rather than to the salvage pathway, as the increase in BH4 could be blocked by the GTPCH inhibitor (synthesis) and not by the DHFR inhibitor (salvage).

Of the incubated vessels, a subset of vessels from three patients was randomly selected and responded well to acetylcholine (Fig 7G). The vasomotor studies were performed to ensure that the vessels were not completely damaged by the prolonged incubation time. The model was used to address the questions of endogenous vs exogenous BH4 (Study I) and the regulation of endogenous BH4 in response to inflammatory stimuli (Study II).

### 4.1.2 Study I

#### *4.1.2.1 Background*

In Study I, 49 patients were randomised to receive low-dose (400 mg/d) or high-dose (700 mg/d) BH4 or placebo for two to six weeks prior to coronary artery bypass surgery. Vascular function was quantified using FMD with MRI before and after treatment, along with plasma BH4 levels. Vascular superoxide, endothelial function and BH4 levels were determined in segments of SV and IMA. Oral BH4 significantly increased BH4 levels in plasma and SV but not IMA. BH2 was also increased in plasma, SV and, if the patient groups of the two doses were put together, also IMA. There was no significant difference in either total vascular superoxide production or NOS-derived superoxide production between placebo- and BH4-treated subjects in either SV ($p=0.7$) or IMA ($p=0.12$). The quantification of vascular function by MRI at baseline and after treatment with either placebo or BH4 revealed no effect of oral BH4 treatment on brachial flow-mediated dilation ($p=0.325$) or arterial stiffness. Samples of SV for an *ex vivo* organ bath displayed no difference in endothelium-dependent relaxation to acetylcholine or endothelium-independent relaxation to sodium nitroprusside between groups (data to be found in the original publication).[74] The *ex vivo* experiments were therefore performed to clarify the fate of exogenous BH4 in vascular tissue.

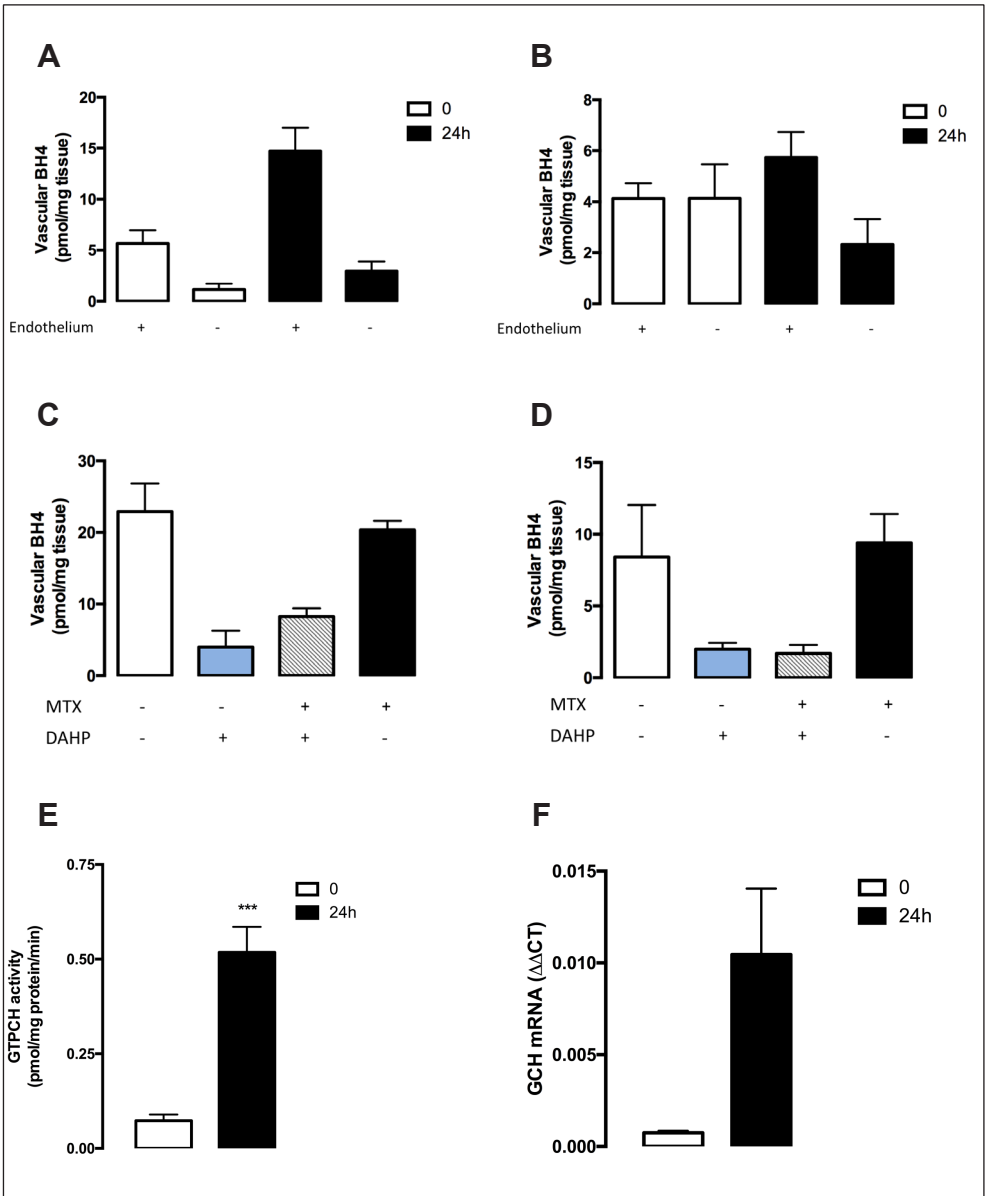


Fig 7. Pilot data from the *ex vivo* vessel model. **A.** Tetrahydrobiopterin (BH4) in saphenous vein (SV) incubated overnight, 24 h, with endothelium (n=24, 24 h vs 0 with endothelium: $p<0.0001$) and endothelial de-nuded (n=5). **B.** Dihydrobiopterin (BH2) in SV with endothelium (n=24, not significant) and endothelial de-nuded (n=5). **C.** BH4 in SV (n=3) in the presence and absence of the GTP cyclohydrolase inhibitor (GTPCH), diamino-6-pyrimidine (DAHP), and an inhibitor of dihydrofolate reductase (methotrexate or MTX). **D.** BH4 in internal mammary artery (IMA) (n=3) after incubation in the presence and absence of inhibitors GTPCH and dihydrofolatereductase (DHFR). **E.** GTPCH activity (neopterin), in SV with endothelium (n=11, $p<0.0001$). **F.** GCH mRNA in SV all layers (n=6).

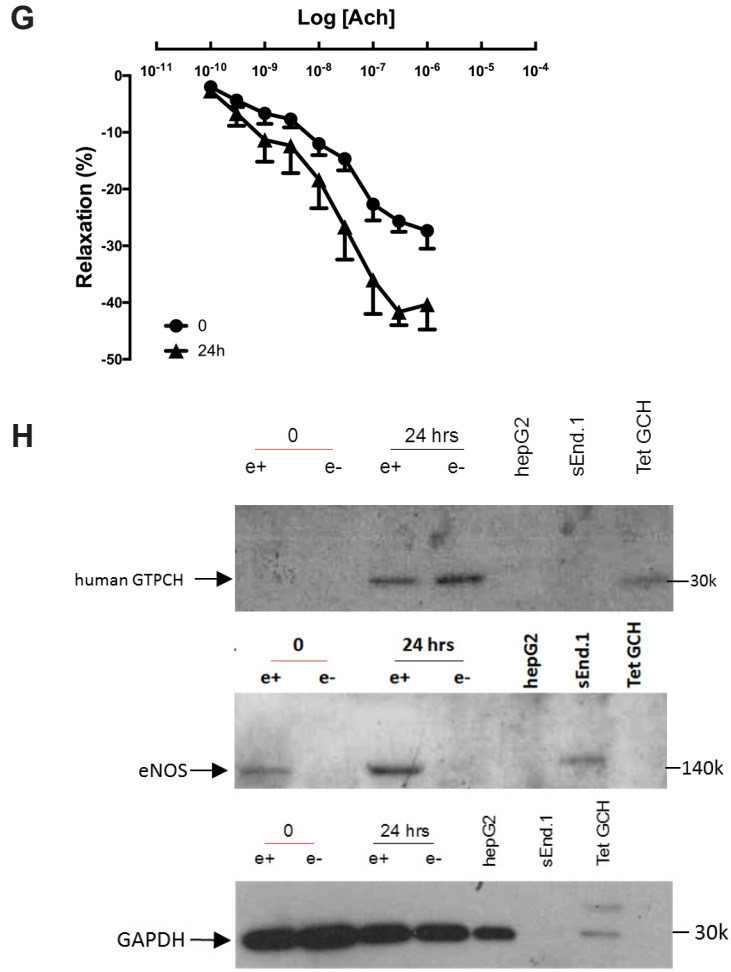


Fig 7 **G.** Relaxation responses in saphenous vein (SV) (0 vs 24 h, n=3). **H.** Western blots of human GTP cyclohydrolase (GTPCH), endothelial nitric oxide syntase (eNOS) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) protein expression loading control. Human GTPCH protein expression in SV. Representative images of n=2. Positive control: hepatocytes (hepG2) and fibroblast cell line with GTPCH over-expression (tetGCH), negative control sEnd.1 cells which expresses only mouse GTPCH. The positive controls were intentionally loaded with less protein due to the large amount of GTPCH proteins in these cells.

*4.1.2.2 Ex vivo experiments*

In order to investigate the fate of exogenous BH4 in human vascular tissue and also to further study mechanistic aspects relating to the influence of oxidation and biopterin uptake, the following experiments were made; segments of SV from an additional six patients were incubated for 30 min under the following conditions: buffer alone (control), BH4 (100nM), BH4 (100nM)+DTE, and BH2 (100nM). Samples of incubation media were taken before and after incubation of the vessel.

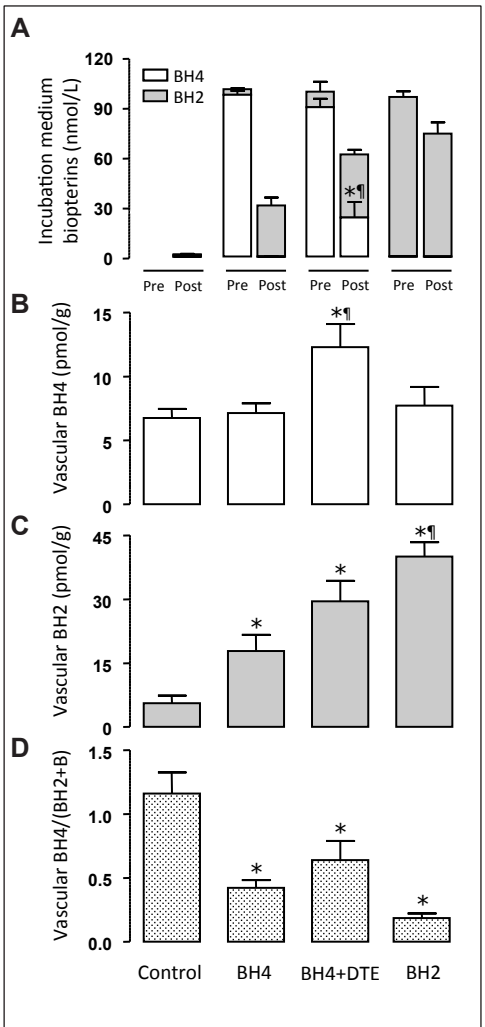


Fig 8. *Ex vivo* incubations of saphenous vein rings (n=6) for 30 minutes with exogenous tetrahydrobiopterin (BH4) examining the uptake of BH4 from the systemic circulation and the impact of oxidation. The conditions were control: buffer alone, BH4, BH4 100 nM plus dithioerythritol (antioxidant) and BH2 100 nM. **A.** Exogenous BH4 was totally depleted in the incubation media, while the addition of antioxidant was able to inhibit the oxidation partially. **B.** and **C.** Incubation with BH4 significantly increased tissue BH2, but there was no increase in tissue. **D.** The ratio of tissue BH4:BH2+B was reduced in comparison to control, even in the presence of antioxidant (values are expressed as mean ± SEM *p<0.05 vs control, and ¶ p<0.05 vs BH4 alone).

The quantification of BH4 in the incubation media revealed that BH4 is depleted by the end of the experiment (Fig 8A). Pre-values showed almost no BH2, which then increased partially towards the end. Vascular BH4 only increased when added in combination with an antioxidant (Fig 8B), incubation with BH4 led to an increase in BH2 levels (Fig 8C) and the BH4/BH2+B ratio (Fig 8D) was reduced in the BH4 group even in the presence of an antioxidant.

### 4.1.3 Study II

#### *4.1.3.1 Background*

To summarise, this study consisted of four parts.

Part 1: 20 healthy individuals were randomised to Salmonella Typhii vaccination (a model of low-grade inflammation) or placebo in a double-blind study. In addition, plasma biopterins, IL-6 and CRP were measured, as well as FMD of the brachial artery using ultrasound. The outcome was a significant increase in IL-6 ($p<0.01$) and CRP ($p<0.05$), as expected, and a significant reduction in FMD of approximately 50% at eight hours ($p<0.01$). The intervention elicited a two-fold increase in plasma biopterins ($p<0.01$).

Part 2: A functional haplotype (X haplotype) in the GCH1 gene, encoding GTP-cyclohydrolase 1, was associated with significantly reduced FMD using ultrasound ($p<0.05$) and high levels of CRP (as an indicator of systemic inflammation) in 440 coronary artery disease patients. The higher the CRP, the poorer the outcome on FMD and higher plasma biopterin levels, if the patient also had the GCH X haplotype.

Part 3: 50 patients identified with coronary artery disease and divided according to GCH haplotype underwent the same inflammatory insult as in Part 1. Briefly, the presence of the GCH X haplotype made it impossible to increase BH4 in response to vaccination and they had a 60% reduction in FMD in comparison with patients without the haplotype (OO).

*4.1.3.2 Ex vivo model*

Part 4: An ex-vivo model was used to evaluate the effect of cytokine stimulation on GCH1 expression, vascular BH4 and relaxation responses to acetylcholine. This ex-vivo model refers to the one described in 3.3.2.

Fig 9 summarizes the findings from the effects of cytokine stimulation on SV and IMA. Endogenous BH4 was significantly increased in SV and IMA which had been exposed to cytokines (TNFα 4ng/ml, IL-6 nM and LPS 80ng/ml) in relation to control and these vessels also had an enhanced relaxation response to acetylcholine. The increase in endogenous BH4 was linked to a significant increase in GCH mRNA expression in SV and IMA. When co-incubation occurred with the GTPCH inhibitor, DAHP, the increase in BH4 or the enhanced relaxation response to ACH could no longer be seen, indicating the critical relationship between GTPCH and BH4 synthesis.

## 4.2 ENDOTHELIN-1 AND EFFECTS ON SUPEROXIDE AND BIOPTERINS

In Studies III and IV, we sought to explore whether ET-1 influences superoxide production and biopterin homeostasis in human vessels.

### 4.2.1 Study III

*4.2.1.1 ET-mediated superoxide production*

A dose-ranging study revealed a marked increase in superoxide production by ET-1 in IMA at 0.1 nM (Fig 10A; $p<0.001$). ET-1 at the threshold dose of 0.1 nM increased superoxide both in IMA (Fig 10B; $p<0.0001$) and in SV (Fig 10C; $p<0.05$). The delta increase in ET-mediated superoxide production was significantly greater in IMA compared with SV (Fig 10D; $p<0.05$). DHE staining revealed an increased signal in all layers of the vessel in comparison to control but more so sub-laminally, suggesting the involvement of smooth muscle cells (Fig 11).

*4.2.1.2 Receptor-mediated superoxide production and sources of ET-mediated superoxide production*

As the next step in the exploration of ET-mediated superoxide production in IMA, we wanted to analyse whether the effect was receptor dependent and the sources of superoxide that were involved. Segments of IMA were incubated in the presence and absence of ET-1 (0.1nM) and inhibitors such as BQ123 ($ET_A$ receptor inhibitor), Dual BQ (BQ123+BQ788, $ET_A$+$ET_B$ inhibitor), L-NAME (inhibitor of NOS), rotenone (inhibitor of mitochondrial ROS), diphenyl-eneiodonium (inhibitor of NADPH oxidase, or flavin-containing enzymes), disodium 4,5-dihy-droxy-1,3-benzenedisulfonate (tiron, superoxide scavenger). The increase in vascular superox-ide was significantly blocked by $ET_A$ and $ET_B$ receptor blockers ($n=15$ vs $n=17$, $p<0.05$), DPI ($n=9$, $p<0.001$) and tiron ($n=6$, $p<0.001$), suggesting that the effect was receptor dependent and that the main source of ET-mediated superoxide production was NADPH oxidase. Fig 12.

### 4.2.2 Study IV

As a continuation of Study III, we sought in Study IV to further explore whether ET-mediated superoxide production involves eNOS. In this study, we used HUVEC, sEnd.1 cells, vessel grafts, an ET-TG mouse model with the overexpression of ET-1 in the endothelium and

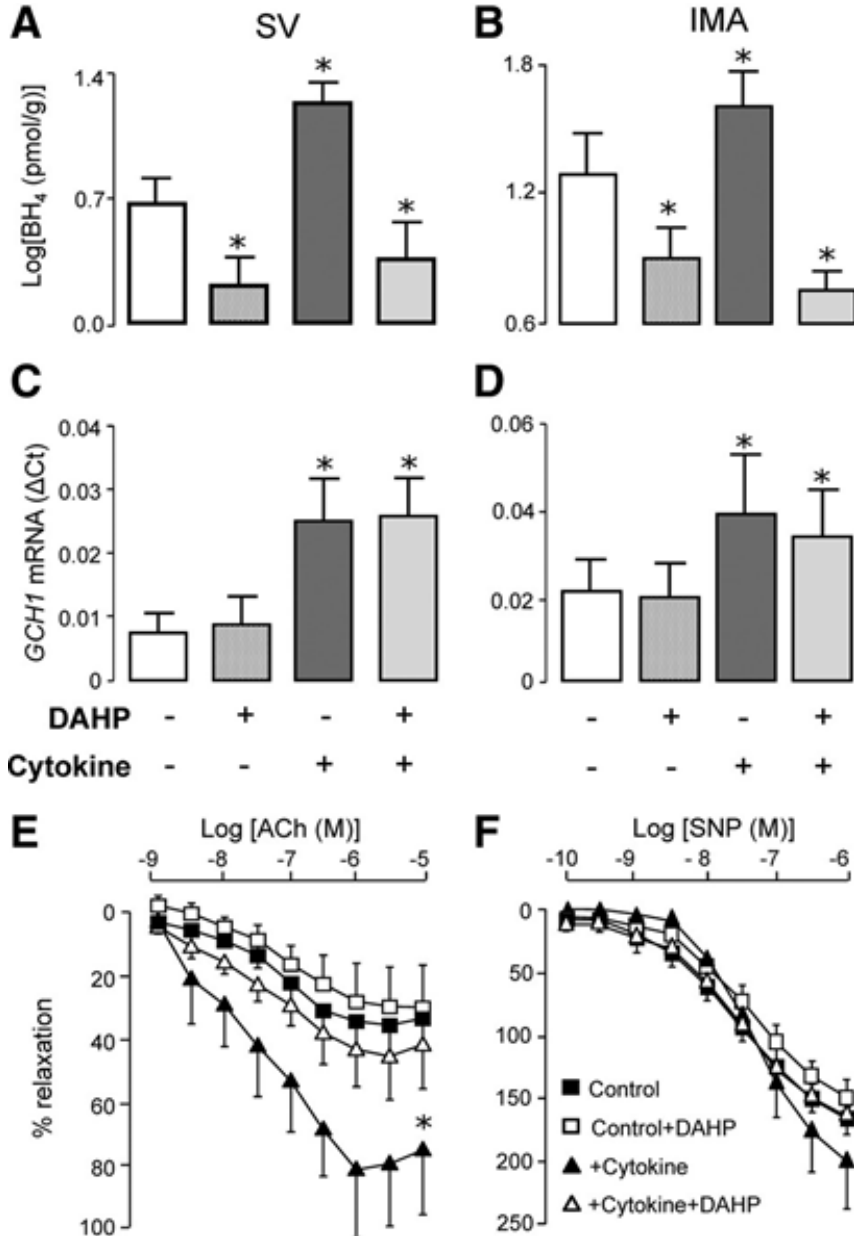


Fig 9. Incubation of saphenous vein (SV) (**A**) and internal mammary (IMA) (**B**) in the absence and presence of cytokine stimulation tumor necrosis factor a (TNFα), interleukin-6 (IL-6) and lipopolysaccharide (LPS) induces a significant elevation in vascular tetrahydrobiopterin (BH4), an effect that was prevented by diamino-6-pyrimidine (DAHP, GTP cyclohydrolase inhibitor). Similarly, cytokine stimulation led to a significant increase in GCH-1 mRNA in both SV (**C**) and IMA (**D**). Cytokine stimulation leads to increased vasorelaxation to acetylcholine (ACH) in SV (**E**) which is reduced in the presence of DAHP. It had no effect on vasorelaxation in response to sodium nitroprusside (SNP) (**F**). Values are expressed as the mean ± SEM *p<0.05 vs control.

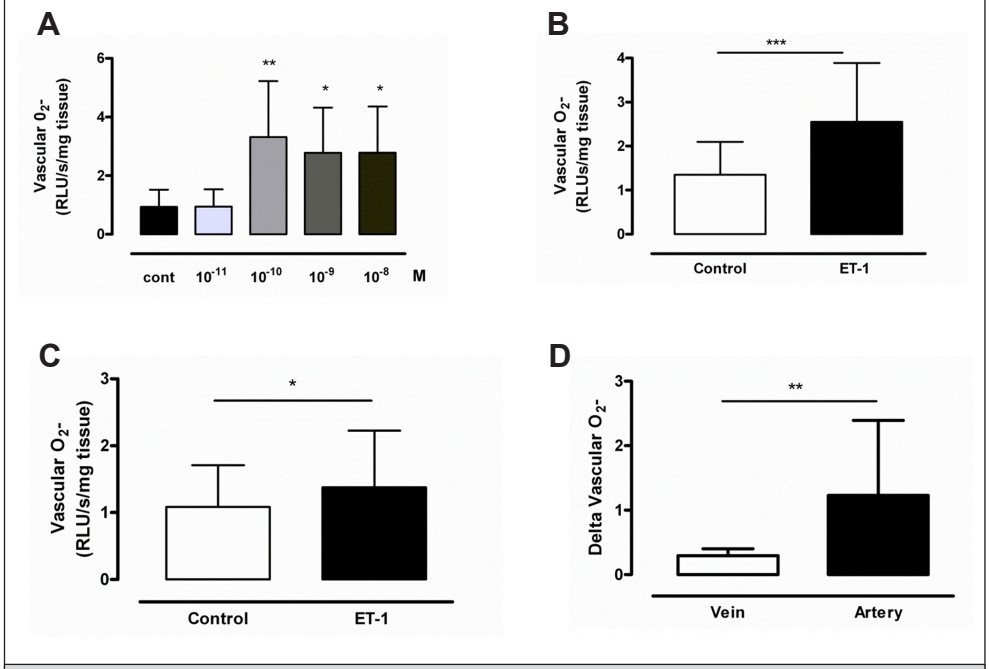


Fig 10 **A**. Dose-ranging study of endothelin-1 (ET-1), in internal mammary (IMA), 45 min, n=8. **B**. Effect of ET-1 (0.1nM) on vascular superoxide ($O_2$-) in IMA, n=33. **C**. Effect on ET-1 (0.1nM) on vascular $O_2$- in saphenous vein (SV). **D**. Delta increase in vascular $O_2$- in IMA vs SV. Values are expressed as the mean ± SD, *P<0.05, **p<0.001,*** p<0.0001.

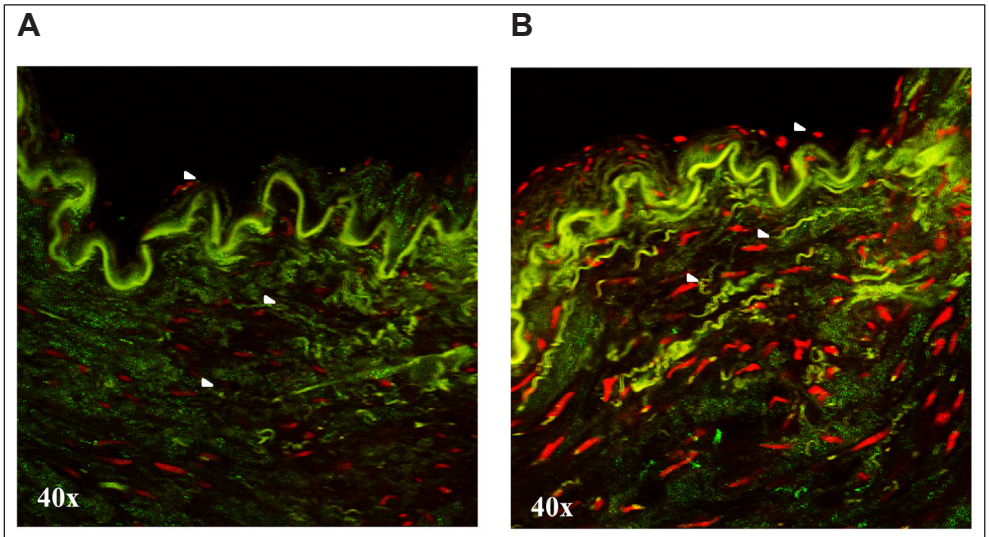


Fig 11 **A**. Internal mammary artery (IMA) control. **B**. IMA+ET-1.
Effect of ET-1 (0.1nM) incubation of vascular superoxide ($O_2$-) in a representative example of IMA (n=3), as determined by dihydroethidium (DHE) fluorescence (red staining indicated with white arrows, green staining represents basal autofluoresence). 40x magnification.

resistance arteries from women undergoing planned caesarean section. This gave us the opportunity to study biopterin homeostasis in many different tissues and models.

We found that, in repeated experiments in HUVEC, there was no significant effect on BH4 or the ratio of BH4:BH2+B following incubation with ET-1 (Fig 13 A: 1.54±1.7 vs 1.68±1.8, p =0.8 and the ratios 2.25±3.32 vs 3.07±4.6; p=0.06). Since HUVEC have very low levels of BH4, at the limit of detection, we wanted to study the possible interaction of ET-1 with eNOS in sEnd.1 cells that have a high expression of GTPCH and BH4. The rationale was to study whether ET-1 influences biopterin homeostasis in a high intracellular BH4 environment. However, there was no significant effect by ET-1 on BH4 or the ratio in sEnd.1 cells (results not shown).

Further, we wanted to investigate whether ET-1 influences biopterin homeostasis in vessels from patients with coronary artery disease. The rationale was that the production of ET-1 in human atherosclerotic vessels is increased and we saw in Study III marked ET-mediated superoxide production. We therefore wanted to explore whether some of this effect was

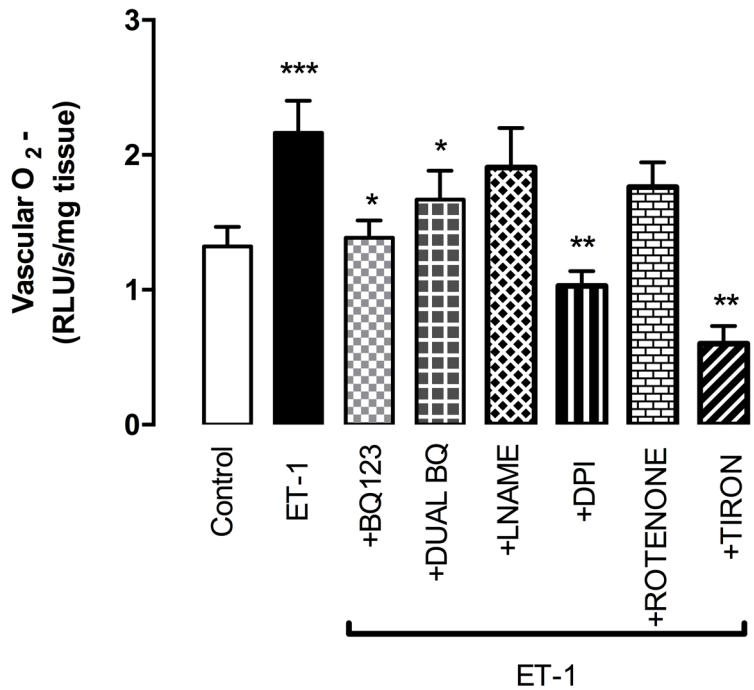


Fig 12. In a series of paired vessel experiments, internal mammary artery (IMA) was incubated in the presence of endothelin-1 (ET-1 0.1 nM) and the following inhibitors of ET receptors BQ 123 and BQ788 and of sources of superoxide $N^G$-nitro-L-arginine methyl ester hydrochloride (L-NAME), diphenyleneiodonium (DPI), rotenone, tiron. Only ET-1 blockade, DPI and tiron were able significantly to inhibit ET-1-mediated superoxide. All values are the mean ± SEM, *P<0.05, **p<0.001,*** p<0.0001.

mediated via direct effects on biopterins. In IMA (Fig 13C; n=10, p=0.4) and SV (Fig 13C, n=12, p=0.8), there was no significant effect from 45 or 240 min incubation of ET-1 (0.1μM) on BH4 levels. Superoxide was also measured in IMA in the presence and absence of L-NAME in order to detect eNOS uncoupling as a source of ET-mediated superoxide production. There was no significant difference in L-NAME inhibitable superoxide between ET-1 (0.1nM) and control (Fig 14A, n=12). We also studied the role of NADPH oxidase and found that NADPH oxidase-dependent superoxide production could be inhibited by BQ123 ($ET_A$ receptor antagonist), in line with earlier findings in Study III (Fig 14B). Furthermore, we investigated whether transgenic mice with ET-1 overexpression in the endothelium and verified endothelial dysfunction had any effect on biopterin homeostasis as a consequence of increased NADPH oxidase activity. In the ET-TG mouse, there was no significant difference

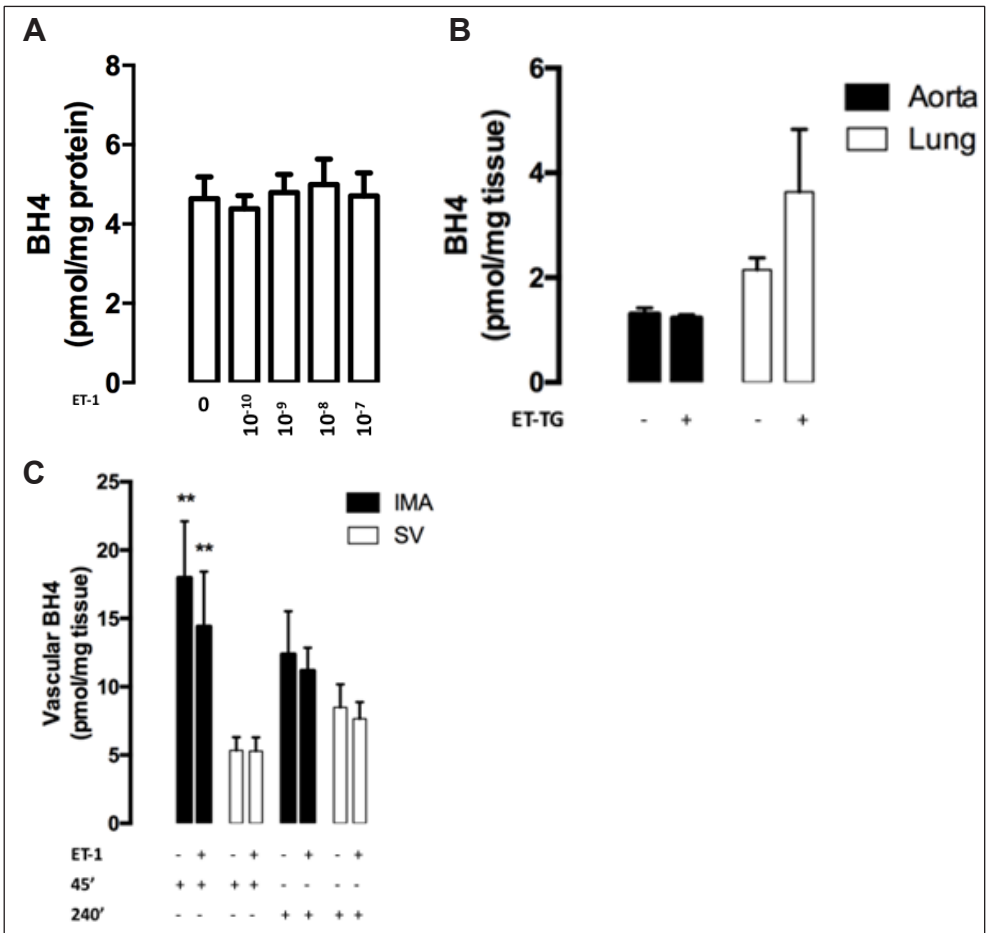


Fig 13. **A.** Quantification of tetrahydrobiopterin, BH4, in human umbilical vein endothelial cells (HUVEC) incubated with endothelin-1 (ET-1) in a dose-ranging study. (n=3 batches, 18 observations). **B.** In ET-transgenic (ET-TG) mice there was no significant difference in vascular BH4 aortas ET-TG (n=18) vs wild type (n=22) or in pulmonary tissue (n=22). **C.** Vascular BH4 in saphenous vein (SV, n=12 for 45 min and for 240 min, n=6) and internal mammary artery (IMA, n=10 for 45 min and n=9 for 240 min) in the presence and absence of endothelin-1 (ET-1). Values are mean ± SEM, **p<0.001 refers to IMA vs SV.

in intracellular BH4 or the ratio of BH4:BH2+B in comparison to WT: aortas, lungs and plasma were examined (Fig 13B).

ET-1 has previously been shown to induce endothelial dysfunction in humans.[41] As a final set of functional experiments, we studied the effect of ET-1 on endothelial function and whether BH4 was able to prevent ET-mediated endothelial dysfunction in human resistance arteries. We observed a striking effect of ET-1 in resistance arteries in subcutaneous fat harvested from pregnant women undergoing caesarian section, with a significant reduction in relaxation to ACH (Fig 14C; n=5, $p<0.0001$) in comparison with norepinephrine (NE, n=6). Endothelial function in these vessels was not restored by BH4 in combination with DTE (n=6), suggesting that this endothelial dysfunction was not mediated by eNOS uncoupling.

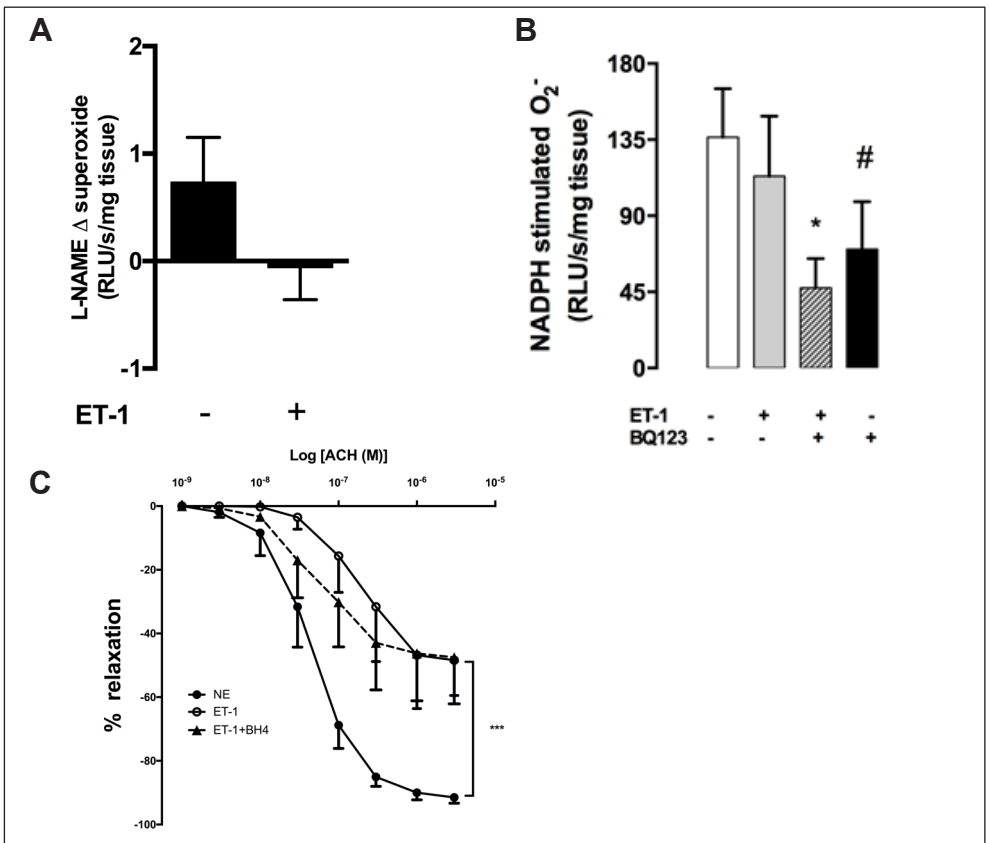


Fig 14 **A**. $N^G$-nitro-L-arginine methyl ester hydrochloride (L-NAME) inhibitable fraction of vascular superoxide in internal mammary artery (IMA) incubated with endothelin-1 (ET-1) for 45 min (n=12, not significant). Determined by lucigenin-enhanced chemiluminescence. **B.** Nicotine amide dinucleotide phosphate (NADPH) oxidase activity in saphenous vein (SV) in the presence and absence of the ET-1 and ETA receptor blocker, BQ123 (n=8, *$p<0.05$ control vs ET-1 + BQ123, and # $p<0.05$: BQ123+ET-1 vs BQ123 alone). **C.** ET-1 in resistance arteries with a significant reduction in relaxation to acetylcholine (ACH) (n=6, $p<0.05$) in comparison with norepinephrine (NE, n=6). ET-1 induced endothelial dysfunction was not restored with tetrahydrobiopterin (BH4), n=5. All values are mean ± SEM. ***$p<0.0001$.

# 5 GENERAL DISCUSSION

The main hypothesis of this thesis was that eNOS and endothelial function is regulated by the availability of BH4.

Four studies were designed with the specific aim of investigating:

(1) Exogenous BH4 and effects on endothelial function in patients with coronary artery disease (Study I)

(2) The regulation of endogenous BH4 and subsequent effects on endothelial function in patients with coronary artery disease (Study II)

(3) The source of ET-mediated superoxide production and the involvement of an ET-receptor-dependent mechanism in patients with coronary artery disease (Study III)

(4) BH4 availability during increased ET-mediated superoxide production and effects on endothelial function (Study IV)

The main findings of this thesis are the following:

- In a randomized placebo-controlled study oral BH4 does not improve endothelial function due to no net change in the ratio of BH4: BH2+B in patients with coronary artery disease.

- Vascular BH4 is linked to improved endothelial function in patients with coronary artery disease. Inflammation in the vascular wall increases the expression of GCH-1 gene leading to improved vascular function. This is a response to systemic inflammation which leads to increased oxidative stress and chronically to endothelial dysfunction. The bioavailability of vascular BH4 is linked to the expression of GCH-1 gene.

- ET-1 increases superoxide in patients with coronary artery disease via receptor-dependent mechanism which involves NADPH oxidase.

- ET-1 mediated superoxide does not have any effect on BH4 availability and does not change the ratio of Bh4: BH2+B in favor for eNOS uncoupling. ET-1 mediated superoxide induced endothelial dysfunction cannot be explained by eNOS uncoupling.

## 5.1 BIOPTERIN UPTAKE AND RECYCLING

The pharmacologial supplementation of BH4 in human have been studied in different settings: acute infusion in the brachial artery[75-77] or coronary artery[78-80] and also as oral administration[81 82]. High doses of BH4 were needed in the intravenous interventions in order to achieve improved endothelial function. It has been questioned whether the high dose is linked to unspecific antioxidant effects of BH4.

Of the oral supplementation studies only one was-placebo-controlled[81], patients received BH4 during 4 weeks and there was an improvement in NO mediated endothelial function, the lowering of plasma F-isoprostanes in patients receiving BH4 is an indication of reduced oxidative stress systemically. However, there was no measurement of vascular BH4 nor any study confirming that eNOS coupling was the causal explanation of improved endothelial function. In reviewing the literature, questions regarding oral BH4 supplementation and links to endothelial function still remain unanswered.

What is the reliability of oral BH4 reaching its target organ, restoring eNOS coupling and improving endothelial function? The target organ in this case is the vasculature and more specifically the coronary circulation, the provided agent an essential co-factor of eNOS, BH4, and through the use of well-validated instruments the read out was assessment of endothelial function. This was the setting of Study I. The findings were that oral BH4 did not reach its target, i.e. did not increase in arteries (in this case mammary arteries) nor was eNOS coupling increased (measured indirectly as reduced superoxide) and there was subsequently also no change in endothelial function as determined by flow-mediated dilation using magnetic resonance imaging (MRI). The story could possibly end here. How does translational thinking, involving *ex vivo* models, assist in the process of understanding BH4 biology? The *ex vivo* vessel model used in this study made it possible to study the mechanistic background to BH4 uptake in the vasculature. The experiments showed that the incubation of BH4 in an in-vitro setting leads to an increase in BH2. The explanation of this is the rapid oxidation of BH4. When BH4 is incubated together with DTE, a potent antioxidant, BH4 can be preserved, but the effect did not include BH2, which increases irrespective of DTE, i.e. oxidation continued and it is possible that the effect of DTE then diminishes over time. In addition to combining BH4 with an antioxidant, there are other possible ways of ensuring the delivery of BH4 via liposomal packaging. A recent ischemic-reperfusion intervention in rat hearts[83] showed a markedly reduced infarct size in rats that had received liposomal BH4, due to reduced oxidative stress and increased NO as measured by staining, suggesting improved eNOS coupling. Although the measurement of NO can be questioned, it is still a promising method to ensure BH4 delivery in a pro-oxidative setting.

As discussed earlier in this thesis, it has been seen in studies with purified eNOS that it is the BH4:BH2+B ratio which is a more important determinant of eNOS uncoupling than absolute levels of BH4.[19] It is therefore not surprising that, although BH4 levels increased substantially in SV, the same thing applied to BH2, resulting in an unchanged ratio and therefore also no effects on eNOS coupling. In this model, we were unable to see that BH2 is able to recycle back to BH4 via DHFR and increase the level of BH4. Although DHFR activity was not measured, there was no increase in vascular BH4 and, in the pilot model of incubations of SV, the main explanation of increased BH4 levels was related to synthesis rather than recycling. In contrast studies with cell cultures, for example, Sawabe *et al.*[84] show that exogenous BH4 is not taken up by the cell, but, if sepiapterin is given, the cell takes up BH2 and increases BH4 through a salvage pathway. This was not seen in the *ex vivo* experiments. Interestingly, the pilot model showed a significant increase in endogenous BH4 in SV but no increase in BH2. Endogenous BH4 does not appear to undergo the same oxidation process as oral BH4. Another question is why there was a significant increase in BH4 in SV but not in IMA. This needs to be studied further to determine whether uptake differs between veins and arteries.

One very important aspect of Study I is the detailed study of the fate of BH4 from circulation (plasma) to vessel, giving new insights into the intricate reality of BH4 uptake, oxidation and recycling. At the same time, some of the limitations are connected to the *ex vivo* experiments, as biopterin biology is not the same and cannot be compared with an *in vivo* setting. For example, the oxidation of vascular BH4 will be different *ex vivo*, the rate of oxidation will probably be higher and the possibility of self-generating superoxide will also be more likely in hyperoxia (which is the case in the organ bath).

## 5.2 INFLAMMATION INCREASES ENDOGENOUS BH4

Cell models have shown that GCH-1 expression is increased by stimulation with inflammatory cytokines: IFNγ[85], TNFα[86], and IL-1ß[87]. When it comes to atherogenesis and the pro-inflammatory systemic influence in patients with coronary artery disease, the complexity increases. A large clinical cohort showed that high plasma BH4 is linked to impaired vascular function due to low vascular BH4[29] and, in transplant patients, vascular injury due to inflammatory stress is related to reduced eNOS expression and increased eNOS activity[88], a paradoxical finding. How do you address the influence of low-grade inflammation on the expression and actions of GCH-1 in humans?

In creative models, Study II addresses this question. Study II is a large multi-faceted study exploring the complex associations between impaired endothelial function, inflammation and plasma and vascular biopterins. The study highlights the role of endogenous BH4 as a vascular defence mechanism and specifically GCH-1 expression in relation to endothelial function and pro-inflammatory stimuli in healthy, diseased and ex-vivo settings.

The novel findings of this study suggest that

(1) Plasma and vascular biopterins are regulated differently in their respective compartments

(2) Plasma biopterins increase in relation to acute inflammation and are correlated with impaired vascular function. The increase in plasma biopterins is due to the increased release of BH4 from hematopoietic cells and the liver and not because of increased BH4 from the endothelium.

(3) Vascular biopterins increase as a direct consequence of increased GCH-1 gene expression. Increased vascular biopterins are related to improved vascular function, but this does not necessarily occur *in vivo* in the setting of atherosclerosis, as patients with coronary artery disease had increased plasma BH4 and impaired endothelial function. It is the inability of the endothelium to increase vascular BH4 during systemic inflammation which is linked to impaired endothelial function.

One important limitation of this study is the artificial inflammatory setting, both *in vivo* with vaccination and *ex vivo* with a cocktail of inflammatory cytokines. Is it comparable and are the results applicable in a clinical setting? For example, in the *ex-vivo* experiments, LPS was used. It is mainly found in cases of septic shock, so do we get the same increase in endogenous BH4 without LPS? This was not studied. However, in the pilot model, it was

clear that the significant increase in BH4 occurred over time without stimulation, so it might be possible that this was due to inflammatory cells in the vascular wall. Further studies would be needed to examine the occurrence of inflammatory cells, the presence of inflammatory cytokines and also the engagement of transcriptional modifications such as the involvement of NF-Kβ, as well as antioxidant defence mechanisms involving nuclear factor erythroid 2-related factor (NRF-2) in an unstimulated vessel.

## 5.3 ENDOTHELIN-1 AND VASCULAR SUPEROXIDE

ET-1 has been found to be increased in patients with coronary artery disease both in plasma and in the expression of endothelin-converting enzyme in atherosclerotic vessels.[41] Moreover, when it comes to certain risk factors such as hypertension, ET-1 has been found to mediate superoxide production, resulting in the reduced bioavailability of NO.[89] Linking ET-1 to oxidative stress is not in any way a new concept. Table 1 summarizes the current studies addressing this link. Our aim in Studies III and IV was specifically to study the role played by ET-1 and oxidative stress in coronary artery disease having the necessary access to vascular tissue, which has not been studied before. Does ET-1 increase superoxide in coronary artery disease and what are the mechanisms underlying this? In contrast to a large set of animal and cell studies, this has not been explored. We found that ET-1 markedly increases superoxide in coronary artery bypass grafts as determined by lucigenin-enhanced chemiluminescence. In order to investigate whether ET-1 mediates superoxide production via a receptor-dependent pathway, we exposed paired segments of IMA to ET-1 in combination with its receptor antagonists, BQ123 ($ET_A$) alone or in combination with BQ788 ($ET_B$). In-vitro studies show that both receptors are able to contribute to superoxide production.[42 45] In our study, we demonstrate a clear reduction in ET-induced superoxide production by selective $ET_A$ receptor blockade and no additional effect of dual $ET_A$/$ET_B$ receptor blockade. These observations suggest that $ET_A$ is the predominant receptor mediating arterial ROS production.

It is not clear which enzymatic source of superoxide causes ET-mediated superoxide production and we therefore sought to study this further. Since the inhibition of basal superoxide has been studied in detail [90], we sought to focus on the inhibition of ET-induced superoxide production. In this study, ET-induced superoxide production is significantly inhibited only by tiron, a superoxide scavenger, and DPI, an inhibitor of flavin-dependent enzymes such as NADPH oxidase, xanthine oxidases and NOS. The specific inhibition of NOS, xanthine oxidases and mitochondrial enzymes did not inhibit ET-induced superoxide$^-$, indicating that it is unlikely that these enzyme systems contribute to ET-induced superoxide production. Accordingly, we conclude that NADPH oxidase is likely to have contributed substantially to the superoxide production that was observed following incubation with ET-1. This is in accordance with a previous report which demonstrated that ET-1 increases vascular superoxide generation via NADPH oxidase in a model of low-renin hypertension [42]. However, in 2005, Ergul *et al*[56]. were unable to detect any increase in NADPH-oxidase-mediated superoxide production following ET-1 incubation in SV. This difference may be due to the fact that frozen and homogenised SV instead of fresh IMA were used. In our study, we observed a significant increase in superoxide production after only 45 min of ET-1 exposure, suggesting the rapid stimulation of NADPH-oxidase activity. To our knowledge, no studies performed on human vessels describe the

source of ET-mediated superoxide production and a possible intracellular pathway in the same study. A previous report shows that ET-1 increases the expression and activity of p47 phox in rat aortic rings via the $ET_A$ receptor, which would suggest that ET-1 is critically involved in the activation of NADPH oxidase.[52] The suggested signalling[52] The signaling pathway was the sequential activation of protein kinase C (PKC), c-Src and ERK ½.

The following limitations were identified; first, the study cohort should ideally include healthy controls, not only because of differences in vascular pathophysiology but also due to ongoing medication such as statins, ACE inhibitors and beta-blockers, which will have specific effects on superoxide generation among patients. Second, the ET-mediated superoxide production was measured in vessels *ex vivo* and the present findings cannot be extrapolated to *in vivo* conditions. However, it is interesting that ET-1 also induces endothelial dysfunction *in vivo* via a mechanism that is related to oxidative processes. [91] Furthermore, ET-receptor blockade using BQ123 and BQ788 improves endothelial function in patients with coronary artery disease.[92 93] Third, the small amount of tissue limits the opportunity for multiple observations in each patient. However, each observation was made from paired samples from the same patient and the study cohort was relatively large.

### 5.3.1 Endothelin-1 and eNOS uncoupling

ET-1 is increased in the vasculature of patients with coronary artery disease[94 95], almost abolishes endothelium-dependent vasodilation in healthy men[96] and induces a marked increase in superoxide production in coronary artery bypass grafts (Study III). It is therefore important to further elucidate the mechanistic explanation behind these actions of ET-1 in order to increase our understanding and to develop new therapeutic strategies to prevent the detrimental effects of ET-1. Since it was suggested that ET-1 mediates superoxide production partly through uncoupled eNOS in the rat aorta [43], we wanted to investigate the link between ET-1 and biopterins in more detail. Although our initial findings showed no effect of eNOS inhibition when measuring ET-1-mediated superoxide, the link between eNOS uncoupling and ET-1 could not be ruled out.

The main finding in Study IV was that ET-1 did not evoke any significant change in biopterins in sEnd.1 cells, HUVEC, ET-TG mice or coronary bypass grafts. Furthermore, BH4 was unable to inhibit ET-mediated endothelial dysfunction in resistance arteries from pregnant women and L-NAME pre-incubation followed by ET incubation did not affect ET-mediated superoxide production in coronary artery bypass grafts. Collectively, these findings suggest that ET-1 does not contribute to eNOS uncoupling in these tissues. One strength of this study was the advantage of using ET-TG mice with ET-1 overexpression in the endothelium. The long-term effects on biopterins of increased local endogenous ET-1 production in endothelial cells could be determined in the aorta, lungs and plasma. However, even though there is a three times higher vascular tissue ET-1 mRNA content and seven times higher ET-1 plasma levels in this model[71], we were unable to see any effects on biopterins in these tissues. This may be due to the fact that this phenotype is relatively healthy without overt atherosclerosis. In addition, oxidative stress and NADPH oxidase are increased in this model which, together with our findings, collectively suggests that ET-1 affects NADPH rather than eNOS uncoupling in this model.

Increased ROS production is a key factor in disease progression and ET-1 contributes to this process, particularly in cardiovascular disease states such as coronary artery disease[97]. In Study III, it was observed that ET-1 was able to increase superoxide production, most likely via NADPH oxidase, in human coronary artery bypass grafts. It is possible that this increase in superoxide production could lead to the oxidation of BH4 and result in eNOS uncoupling or, alternatively, that ET-1 could have direct effects on biopterin synthesis via the induction of GTPCH. Zheng *et al*.[46] describe a low renin hypertension model in rat, where exogenous ET-1 increases superoxide production in carotid arteries. In addition, in this model, L-NAME does not inhibit ET-induced superoxide production. Only apocynin and ET-receptor antagonism are able to inhibit this increase, indicating that the main source of superoxide induced by ET-1 is NADPH oxidase[46], which was also the conclusion of Studies III and IV.

# 6 CONCLUSIONS

In this thesis we have studied the mechanisms of eNOS regulation in endothelial dysfunction from bedside to bench or intracellular level, studied the very key factors regulating eNOS uncoupling in the setting of patients, mouse models and endothelial cell models.

In summary I conclude the following:

1- exogenous BH4 (oral) does not improve endothelial function due to increased levels of BH2 as a direct consequence of increased oxidation of BH4 and no net change in the ratio of BH4: BH2+B in patients with coronary artery disease.

2- high levels of vascular BH4 is linked to improved endothelial function in patients with coronary artery disease. Chronic systemic inflammation leads to increased oxidative stress and endothelial dysfunction. Acute inflammatory insult on the vascular wall leads to increased GCH-1 gene expression and increased vascular BH4 which in turn results in improved vascular function. This response is not seen in patients with deficient GCH-1 due to mutation in the GCH gene (GCH 1 X haplotype).

3- ET-1 increases superoxide production in patients with coronary artery disease via a receptor-dependent mechanism which involves NADPH oxidase.

4- ET-mediated superoxide does not change BH4 availability in the studied tissues and does not increase BH2. Endothelial dysfunction induced by ET-mediated superoxide production can therefore not be explained by eNOS uncoupling.

# 7 FUTURE PERSPECTIVES

During the course of my research, I wanted to address the question of oxidative stress in endothelial dysfunction, with the broader perspective of possibly shedding light on optimizing the treatment of patients with cardiovascular disease. Large antioxidant trials have failed to produce any outcome on hard end-points such as death or myocardial infarction. There are many review articles summarizing these neutral results[98]. Why then is it important to study oxidative stress? Is there any justification for antioxidants in the treatment of patients with coronary artery disease?

The role of oxidative stress becomes clear in the light of the importance of identifying and treating patients at risk of myocardial infarction. These are patients with endothelial dysfunction and other risk factors such as hypertension, hypercholesterolemia, diabetes and smoking. With improved knowledge of oxidative signaling, we can use the key enzyme systems as markers which will contribute to the diagnosis, prevention and treatment of cardiovascular disease. In a recent review, several important enzyme systems were identified. They could possibly be used in a clinical setting as redox biomarkers[99], among these there is also the role of BH4 as a potential biomarker reflecting overall vascular redox status. ET-1 is without doubt an important peptide in the progression of disease and, with current and future studies, it will be possible to foresee combinations of ET blockade with, for example, blockers of downstream signaling molecules involving ROS.

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